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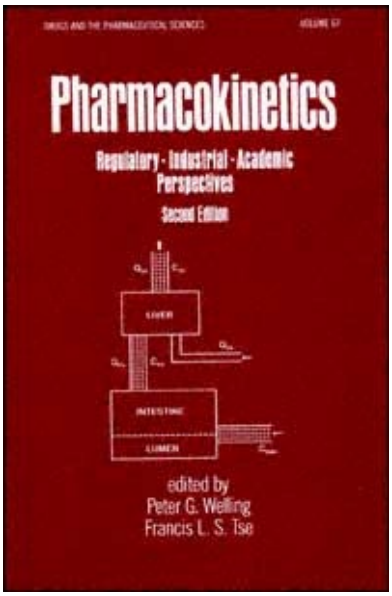

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Pharmacokinetics

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Pharmacokinetics

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To Caroline, Christine, Clara, Graham, Luisa, and Stephen

Preface

The objective of this book is to present the latest concepts and developments in the area of pharmacokinetics from the perspective of those actively working in the regulatory, industrial, and academic environments. The first edition of the book was published in 1988. Since that time there have been considerable upheavals in the pharmaceutical industry. These have been associated with quantum scientific advances and achievements as well as continued globalization of pharmaceutical drug discovery and development on the one hand, and the challenges of cost containment and increasing regulatory demands on the other. These events continue to have a considerable impact on drug development and also on the critical role that pharmacokinetics and drug metabolism play in discovery and development and in drug therapy.

We felt it both timely and necessary to prepare this second edition in order to keep abreast of the changing roles and responsibilities of pharmacokinetics. This book is the result of that initiative. While the overall thrust of this second edition is similar to that of the first edition, a number of changes have been made. Although a strong component of classical pharmacokinetics and metabolism has been retained, there is greater emphasis on topics that are rapidly changing within the dynamic framework of the application of pharmacokinetic principles.

The first chapter addresses the central issue of good laboratory practice regulations, as applied to pharmacokinetics. This is followed by chapters on methods of assessing drug absorption, drug delivery systems, peptide and protein drug de-

livery, and membrane transport. Drug distribution is addressed in chapters on blood-brain barrier permeability, pharmacokinetic and pharmacodynamic relationships of drugs acting on the central nervous system, and quantitative whole-body autoradiography. Two chapters discuss recent advances in drug metabolism methodology and the use of hepatic in vitro systems as models for human drug metabolism. The latter part of the book focuses on drug development with chapters on the monolithic approach of integration of pharmacokinetics into development, nonclinical and clinical pharmacokinetics in drug discovery and development, and population pharmacokinetics and pharmacodynamics. The book concludes with regulatory perspectives of bioavailability and bioequivalence of oral controlled-release products, and statistical issues relating to bioavailability and bioequivalence studies.

Our intent in preparing this book has been to continue to present the latest concepts in the most rapidly changing areas in the broad discipline of pharmacokinetics. We have drawn from the expertise of authors who are active leaders in their respective fields, and have attempted to focus on areas that are undergoing the greatest change and are having the greatest influence on the many applications of pharmacokinetics and drug metabolism. We hope that this second edition of pharmacokinetics has achieved this objective. We are grateful for the generous contributions of our colleagues, who took the time from their busy schedules. We also thank Theresa Davis for her limitless administrative help and patience during the preparation of this book.

PETER G. WELLING
FRANCIS L. S. TSE

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1
Principles of the Good Laboratory Practice Regulations Applied to Pharmacokinetics

Dexter S. Goldman
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I. History and Intent of the Regulations

A. 19701978

All individuals, and individuals is used in its broadest sense, within a social system are bound by regulations, spoken as well as unspoken. Within political units, organizations are bound by regulations, some of which may make good sense, others of which are used to exemplify and even drive social policy. In the 1970s in the United States the Food and Drug Administration (FDA), rocked by allegations and proof of misconduct and fraud within segments of the pesticide* and drug testing industry, used its authority under the Federal Food, Drug and Cosmetic Act (FFDCA) and the Public Health Service Act (PHSA) to propose regulations governing the conduct of nonclinical laboratory studies** [1] submitted to the FDA by manufactures and other petitioners to demonstrate the safety and effectiveness of their products. Historically, the FDA never has had the resources to test or evaluate the safety and effectiveness of chemicals under its jurisdiction. Public policy was, therefore, to require manufacturers and petitioners to provide these data at their own expense; this policy continues to this day. The FDA prescribed the type and extent of such testing, reviewed the data submitted, and eventually either permitted distribution and sale of a regulated chemical or device or, lacking sufficient information, refused to allow distribution and sale.

Prior to 1978 the FDA had in place a program of small size that was used as an adjunct to desk audits and reviews of data supporting petitions for action. These on-site investigations were generally the result of questions raised during the review of data; that is, they were retrospective. The FDA had assumed that the nonclinical studies submitted were the result of the application of appropriate testing procedures by a testing facility. The change came in 1975 when FDA investigators showed that fraud and deception had entered the testing industry. FDA's point of view is best illustrated by an understated analysis of the problem that appeared in the preamble to the proposed rule on Good Laboratory Practice (GLP) regulations for nonclinical studies [2]:

> Recent FDA experiences have identified significant problems in the manner in which nonclinical laboratory studies are being performed. Deficiencies were found during inspections of the testing facilities of major pharmaceutical firms, inspections of several private contract testing facilities, and internal reviews of

*The toxicological properties of pesticides were tested since the FDA classified these chemicals as food additives and, accordingly, they were under its jurisdiction.

**Nonclinical laboratory study means in vivo or in vitro experiments in which test articles are studied prospectively in test systems under laboratory conditions to determine their safety. The term does not include studies utilizing human subjects or clinical studies or field trials in animals. The term does not

include basic exploratory studies carried out to determine whether a test article has any potential utility or to determine physical or chemical characteristics of a test article.

> toxicity studies of color additives conducted by FDA. How widespread or serious the problems are is not known at this time. The agency is concerned, however, that significant deviations in the quality and integrity of reported data might be of a greater magnitude and scope than had previously been assumed.

The preamble then lists the important deficiencies that had been observed, including:

Experiments that had been poorly conceived, carelessly executed, or inaccurately analyzed and reported

Technical personnel unaware of the importance of protocol adherence, accurate observations, accurate administration of the test substance, accurate recordkeeping, and record transcription

The lack of management review of data or supervision of employees

Protocol design that did not allow the review and analysis of all the data

Lack of assurance of the scientific qualifications and adequate training of study personnel

Disregard for proper laboratory, animal care, and data management procedures

Lack of study oversight by sponsors when the study was conducted by a contract testing facility

Lack of verification of the completeness and/or accuracy of the data reported to the FDA

As a result of these revelations, hearings were held before the United States Senate Subcommittee on Health of the Committee on Labor and Public Welfare and the Subcommittee on Administrative Practice and Procedure of the Committee on the Judiciary during the period of July, 1975, to July, 1976. Dr. A.M. Schmidt, the commissioner of the FDA, summarized the problems and expressed concern about the absence of industry-wide standards for conducting animal or other test system studies intended to be submitted to the FDA. The rest, in ordinary parlance, is history. The Congress gave the commissioner authority to issue the necessary regulations, for the first time including the term good laboratory practice regulations modeled after the good manufacturing practice regulations already in force. The Congress also gave the FDA a large appropriation ($16.5 million) effective October 1, 1976, for the purpose of obtaining and utilizing the resources needed for this new program.

B. The Regulations Since 1979

The GLP regulations have remained remarkably constant over the past 17 years. Their weakest point, namely their lack of specificity in many regulatory details, has proved in

the long run to be their strength in that each testing facility can interpret many aspects of the regulations in consideration of its resources, type of testing, physical plant, and so on. The regulations have grown by evolution, not by additional regulations or pronouncements.

Although the scope and interpretation of the GLP regulations have generally enlarged over the past decade, the intent remains the same: ensure the quality and integrity of the data that support applications for research and marketing permits for products regulated by the FDA. The quality of the data can be expressed in terms of high standards, careful planning, and other such terms. The integrity of the data means that all data are reported, that the original observations have been recorded with a clear record of ownership, that the study can be reconstructed from the raw data, and that there can be no undocumented changes to the data. Data integrity, originally seen as having the backing of traditional notebooks, extends to all means of data entry and recording, including computerized data acquisition and manipulation.

The Organization for Economic Cooperation and Development (OECD) recognized early on that this new initiative on the part of the FDA would have to be matched by similar regulations in member countries. The OECD issued its guidelines on GLPs in 1981 and over the years member countries have put into place the needed legislation and other aspects of regulatory control of study management needed to bring all nonclinical laboratory studies into compliance with GLP regulations.

The United States Environmental Protection Agency (EPA) issued its own GLP regulations a few years later. While originally attempting to avoid issuing new regulations by inserting study management principles into the various test guidelines, the effort failed and GLP regulations were issued in 1983 for the Toxic Substances Control Act (TSCA) [3] and the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) [4]. An administrative decision was made to promulgate regulations that, with minor differences, adopted the FDA regulations word for word. This was done even though the testing requirements as well as the scope of testing required under both TSCA and FIFRA were markedly different from those of the FFDCA. This decision effectively removed a large percentage of critical studies from the scope of EPA's GLP regulations, an oversight that was not corrected until 1989 [5].

Today, across many countries, there is only one agreed-upon standard for the management of nonclinical studies as well as the integrity of the supporting data, and that standard is the GLP regulations. This is the standard against which most, if not all, studies are being judged, whether or not the study falls within the scope of the regulations. Herein lies the problem with regulatory control and pharmacokinetics.

II. Applicability to Pharmacokinetics

The applicability of the principles and practices of the GLP regulations to pharmacokinetic studies may depend on where you conduct the study and to which

regulatory authority you submit the study.

In the United States it is very clear that the FDA considers pharmacokinetic studies to be outside the scope of the GLP regulations. FDA considers pharmacokinetic studies to be *supportive of* required health and safety studies and not as health and safety studies submitted in support of a research or marketing permit.* Accordingly, if your FDA marketing permit is for the US domestic market only, GLP regulatory compliance is not required for pharmacokinetic studies. However, a problem arises when the studies are reviewed. In the absence of any other quality guidelines, the FDA will review pharmacokinetic studies against the standards set by the GLP regulations. For this reason many pharmaceutical companies in the United States have voluntarily imposed GLP regulatory compliance on pharmacokinetic studies.

The regulatory situation in the European Community remains confusing. The basic framework for regulatory compliance lies in Council Directive 75/318/EEC [6]. This directive was expanded and direct reference was made to pharmacokinetic studies in 1991 [7]. Pharmacokinetics is considered as part of the overall toxicity testing program that may be ordered by member states. In the United Kingdom the Department of Health has stated that any study used in support of a safety submission must be conducted in compliance with the (UK) GLP regulations. Germany, on the other hand, considers such studies outside the scope of the GLP regulations. Many German companies have introduced GLP compliance for pharmacokinetic studies on a voluntary basis.

In Europe, member states were required to bring Council Directive 91/507/EEC [7] into force no later than January 1, 1992. Part 3 of this directive, Toxicological and Pharmacological Tests, lists pharmacokinetics at Part II.G as:

> Pharmacokinetics means the study of the fate of the active substance within the organism, and covers the study of the absorption, distribution, biotransformation and excretion of the substance.
>
> Information on distribution and elimination (i.e. biotransformation and excretion) shall be necessary in all cases where such data are indispensable to determine the dosage for humans, and in respect of chemotherapeutic substances.
>
> Pharmacokinetic investigation of pharmacologically active substances is necessary.

All such investigations are included in the general requirement stated at Part 3,I.1: Member States shall ensure that safety tests are carried out in conformity

*The United States EPA, on the other hand, requires certain pharmacokinetic (metabolism or nature of the residue) studies for certain pesticides. These requirements are detailed at Title 40 Code of Federal Regulations, Part 158. Since these are required studies they, along with all other EPA-required studies, must be conducted in full compliance with the GLP regulations.

with the provisions relating to good laboratory practice laid down by Directives 87/18/EEC and 88/320/EEC.

The discussion now devolves to the meaning of safety tests. The Association of the British Pharmaceutical Industry proposed, in October, 1992, that the paragraph on safety tests be amended to exclude pharmacological, pharmacodynamic, and ADME studies that do not form an integral part of a toxicity study. Again, the decision hinges on the relationship between the pharmacokinetic and the toxicity studies and it was proposed that the decision should be left to the discretion of the individual manufacturer. It is unlikely that this argument, based on the purported additional cost burden to the manufacturer, will carry significant weight over the long run although it might be invoked for an interim period. What is more likely to happen is a decision by the EC that any *required* study must be conducted in full compliance with the GLP regulations of the member state, and that any *voluntary* (i.e., research and development [R&D]) pharmacokinetic study would be excluded from the GLP requirement. Since true discovery or R&D studies have always been excluded from the GLP regulatory requirements, this seems to be a reasonable compromise. However, as described above, it carries within it the seeds of its own destruction: any expert review of the dossier must identify any noncompliant studies in the dossier and describe the importance of that study to the acceptance of the dossier, thus closing the circle again. There is one standard of study performance and data integrity accepted worldwide and that is a study conducted in compliance with the GLP regulations. This is how any noncompliant study, no matter how excellent in form and content, will be judged. While this particular issue may be discussed for some time, the result appears to be clear. It remains difficult to understand why such a costly and intricate study as pharmacokinetics would be conducted by any manufacturer in a manner not in compliance with these simple and logical principles of study management and data integrity. The key issue may not be complete regulatory compliance; the issue may be the general application of the relevant principles of the GLP regulations.

III. Basic Principles of the Regulations.

In the text that follows there are quotations, indented in the text, from the Good Laboratory Practice regulations. Any such quotations are followed by the specific reference within Title 21 CFR 58.

A. General Provisions

1. Scope of the GLP Regulations

The FDA defines the scope of the GLP regulations as:

This part describes good laboratory practices for conducting nonclinical laboratory studies that support or are intended to support applications for re-

> search or marketing permits for products regulated by the Food and Drug Administration, including food and color additives, human and animal drugs, medical devices for human use, biological products, and electronic products 21 CFR 58.1(a)

and the purpose of these regulations (loc. cit.) to assure the quality and integrity of the safety data filed pursuant to sections of the Federal Food, Drug and Cosmetic Act

2. Definitions

A nonclinical laboratory study is defined as:

> in vivo or in vitro experiments in which test articles are studied prospectively in test systems under laboratory conditions to determine their safety. The term does not include studies utilizing human subjects or clinical studies or field trials in animals. The term does not include basic exploratory studies carried out to determine whether a test article has any potential utility or to determine physical or chemical characteristics of a test article. 21 CFR 58.3(d)

and the term test article is defined as any food additive, color additive, drug, biological product, electronic product, medical device for human use, or any other article subject to regulation under the act or under sections 351 and 345-360F of the Public Health Service Act (21 CFR 58.3[b]).

3. Grants and Contracts

The sponsor of a nonclinical study may contract the study, or part of the study, to another individual or testing facility. In so doing, however, the sponsor retains full responsibility for the conduct of the study as well as the compliance status of the contractor or subcontractor. To that end the sponsor is required to notify the consulting laboratory, contractor, or grantee that the service is part of a nonclinical laboratory study that must be conducted in compliance with the provisions of this part (21 CFR 58.10).

4. Inspection of a Testing Facility

> A testing facility shall permit an authorized employee of the Food and Drug Administration, at reasonable times, and in a reasonable manner, to inspect the facility and to inspect (and in the case of records also to copy) all records and specimens required to be maintained regarding studies within the scope of this part 21 CFR 58.15(a)

The FDA no longer provides advance notice of a regulatory inspection and will in general shift around to a certain degree the quarter of the year in which a testing facility has come to expect its biennial inspection. Reasonable times is generally taken to mean within the scope of the facility's ordinary working hours. Reasonable manner remains undefined.

A refusal on the part of a facility to permit an authorized inspection is today quite uncommon perhaps because the consequences of such a refusal are indeed dire: The Food and Drug Administration will not consider a nonclinical laboratory study in support of an application for a research or marketing permit if the testing facility refuses to permit inspection (21 CFR 58.15[b]).

The implications are clear. If the facility refuses an inspection and the inspection was appropriate, any FDA-related study being conducted at that facility is lost to each and every sponsor of studies at that facility. One can only imagine what will be the future of such a facility. FDA compliance inspections will be discussed at the end of Section III.I.

B. Organization and Personnel

This part of the GLP regulations deals with several crucial concepts related to compliance with the regulations, such as the appropriateness of personnel participating in a nonclinical study, the role of management, the duties of a study director and, the new concept, put in place by the FDA, of the establishment and operations of a functional Quality Assurance Unit (QAU), all within the context of nonclinical laboratory studies.

1. Personnel

An FDA inspector will ask this rhetorical question, Why is this individual performing a part of this nonclinical study? The question will not be asked in this fashion, but implications are clear. Are you prepared to demonstrate that an individual participating in a nonclinical laboratory study has that combination of education, experience, and training to conduct, in an effective and appropriate manner, his or her portion of the study?

The proposed rule on Good Laboratory Practices included professional requirements for certain scientific personnel; these were removed from the final rule. All countries that have issued GLP regulations have the requirement that Each individual engaged in the conduct of or responsible for the supervision of a nonclinical laboratory study shall have education, training and experience, or combination thereof, to enable that individual to perform the assigned functions (21 CFR 58.29[a]).

No further guidance is provided within the regulations and each facility must figure out for itself how to meet the intent as well as the word of the regulations. Education or experience is generally not a problem, this is usually found within the individual's curriculum vitae (c.v.) provided as part of a job application. This c.v. should be periodically reviewed by the individual and brought up to date as needed. Training, on the other hand, is generally more difficult to establish and is often neglected by facility management. An individual may be employed and receive all his or her training on the

job. An individual may be employed in recognition of his or her expertise in a certain field and is useful

to train other employees. Regardless of the training already possessed by an employee, there is always training associated with a new employee's first months on the job. This in-house training must be documented. Documentation may consist of attendance at an employee training session on safety, disposition of biohazardous material, how to use the computer system, or other subjects. Documentation may include certificates of completion of training on vendor-supplied equipment, or an in-house or outside training course on the basics of the Good Laboratory Practice regulations. These are relatively simple to document. In-house training on the use of standard equipment, on manipulative techniques with experimental animals, how to prepare standard solutions, and so on, can easily be documented by using your index of standard operating procedures (SOP). Add, for example, to each title of an SOP a column with the date and initials of the individual being trained to show that he or she has read and understands the SOP and another column for the supervisor to document that the individual has conducted that procedure and can do it without further supervision. This is a simple way to develop an acceptable training record.

Some facilities maintain an instrument log book with signatures of individuals trained to use that instrument. Some facilities document that an individual can perform a pharmacokinetics study on rats: the entire study, from obtaining the test article through chromatographic identification of the metabolites. Any method of documenting training that you wish to use is acceptable as long as you have the training system documented and you follow your own training procedures. You remain the best judge on the adequacy and effectiveness of training.

Documentation of training serves another purpose: in-house promotions and advancement. The training record follows an employee as he or she moves within the company and provides valuable information on an individual's capabilities.

2. Management

Without minimizing management's specific responsibilities for compliance with the GLP regulations (21 CFR 31), it is fair to say that management is in general responsible for achieving all aspects of compliance with the regulations. Management sets up the structure that makes compliance possible; study personnel implement this by following the specific portions of the regulations. The importance of management* is emphasized since it is management that receives the Notice of Noncompliance or Regulatory Letter when problems are found in the testing laboratory.

For each nonclinical laboratory study, testing management shall appoint a study director and, if necessary, replace the study director during the conduct

*Management remains undefined in the GLP regulations, both domestic and foreign.

of the study. Management should further consider naming another individual to take the place of the study director at such time that the study director is unavailable for any period of time because of illness, vacation, professional meetings, and so on. If you do this, find some name other than Assistant Study Director or Acting Study Director, since some inspectors might consider this as having two study directors for the same study, an act that is not permitted.

At this point the FDA put into place the most meaningful and totally new section of the GLP regulations by requiring that for each nonclinical laboratory study, testing management shall ensure that there is a (functional) QAU as defined and described below. I have added functional to this requirement because only recently have many facility managers awakened to the fact that having a QAU is not what the regulations require. Listening to the QAU, making certain that deviations from the regulations are communicated to the study director, and that corrective actions are taken are the hallmarks of a functional QAU that is actively supported by management. This is what FDA means when it writes that management shall assure that there is a quality assurance unit as described in 58.35 (21 CFR 58.31[c]) and assure that any deviations from these regulations reported by the quality assurance unit are communicated to the study director and corrective actions are taken and documented (21 CFR 58.31[g]).

Please note the implication here. The QAU is a staff function of management reporting to management and not to the study director, although there is one exception to this: Any problems found during the course of an inspection which are likely to affect study integrity should be brought to the attention of the study director and management immediately (21 CFR 58.35[b][3]).

The qualifications for QA personnel are the same as for all other technical personnel: the appropriate combination of education, experience, and training for the position.

Management may not delegate the responsibility described above to the study director, although experience shows that many managers make such a delegation and in so doing take themselves out of the QA loop. Such action leaves a manager unaware of what is going on in the facility. These are the same managers who express surprise when a regulatory letter or a notice of adverse findings is addressed to them from the FDA. A proper course of action combines both elements: awareness of compliance problems within the facility along with the knowledge that the study director has received the same written QAU report and will report the corrective actions to management. In practice, it makes little difference if management receives the QAU phase monitoring report directly, passes it to the study director, and receives it back to approve the actions or solutions proposed by the study director or, in another manner, receives the QAU report at the same

time it is given to the study director and then approves the study director's actions or solutions. Both courses of action are acceptable. The course of action chosen will be detailed in the SOP on QAU reporting procedures.

3. The Study Director

The study director is appointed by management based on his or her education, experience, and training. The study director has overall responsibility for the technical conduct of the study as well as for the interpretation, analysis, documentation, and reporting of results. While he or she may delegate technical responsibilities for certain aspects of a study to another specialist, the study director remains the single point of study control and responsible for signing the study report. During the progress of the study the study director, not the QAU, has the additional responsibility of ensuring that (A)ll applicable good laboratory practice regulations are followed. (21 CFR 58.33[f]).

While not specifically detailed in this section of the regulations, it may be inferred from the discussion of the responsibilities of management that the study director must respond to any deficiencies in compliance reported by the QAU, correct the deficiencies noted, and inform management of the correction.

4. The Quality Assurance Unit.

FDA probably did not realize in early 1977 that by describing the functions of the QAU it was establishing a new profession. This is not to say that many facilities were not generating data of integrity and quality, only that a new profession was born and added to the staff of management. The QAU statement (21 CFR 58.35[b][7]) is unique in these regulations: it is the only required signature statement assigned by regulation to an individual. A signature is inferred when the regulations list the content of a final report, but there is no direct mention of this nor is the signer of the report identified. A statement of compliance is likewise required for nonclinical studies, but again the regulations do not specify who is to sign this statement.

The primary function of the QAU, assigned by the regulations, is to:

> Inspect each nonclinical laboratory study at intervals adequate to assure the integrity of the study and maintain written and properly signed records of each periodic inspection showing the date of the inspection, the study inspected, the phase or segment of the study inspected, the person performing the inspection, findings and problems, action recommended and taken to resolve existing problems, and any scheduled date for reinspection (21 CFR 58.35[b][3]).

The message here is ambiguous. I stated earlier that management was responsible for communicating deviations from these regulations to the study director and ensuring that corrective actions are taken and documented. Now we find a section of the regulations, quoted above, that appears to instruct the QAU to insert itself into the corrective action by recommending actions to correct compliance problems and recording the actions taken; this is supposedly a responsibility of the study director, not the QAU. The wording, action

recom-

mended, is ambiguous and may be interpreted in more than one way. Since this part of the regulations has not been changed since 1979, it may be assumed that facilities as well as the FDA are comfortable with a process that has the QAU reporting compliance problems to management and to the study director, and then working with the study director to resolve existing problems. All of this is finally reported to management so that the monitoring file can be closed by the QAU. When such a system is supported by management, compliance problems are found, corrected, and documented through a nonadversarial process.

The QAU has many other responsibilities including maintaining copies of all current and active study protocols, ensuring that any deviations from procedures are documented, and reviewing the final study report for accuracy and completeness. The FDA considers data auditing as quality control, not quality assurance, and will look closely at the ratio of time spent in monitoring studies and time spent in reviewing data and reports. Although there is no direct measure of this ratio to be followed, the sense within both the testing industry and the FDA is that the primary function of the QAU is to monitor studies and report to management on monitoring; at least half of the QAU time should be spent in this function, with the remaining half spent on all its other responsibilities plus teaching and training, a function commonly assigned to the QAU.

The FDA maintains statistics on the percentages of laboratories that have shown major deficiencies in compliance with the GLP regulations. Over the past decade there is a consistent 30% of laboratories with major deficiencies in the QAU. This does not mean that the same laboratories account for this 30% of noncompliance but that this is the percentage of inspected laboratories year after year. To have this occurring 14 years after the regulations were made final, together with many study rejections for noncompliance on the record, seems to indicate an appalling indifference on the part of many facility managers to the need for compliance and the central position of the QAU in achieving and maintaining compliance with the GLP regulations.

C. Facilities

The intent of this subpart this section of the regulations is to ensure that the facilities in which nonclinical laboratory studies are conducted are adequate for the purpose of the studies.

1. Animal Care Facilities

This is not a general problem for pharmacokinetic studies, since the animal facilities employed in such studies are minimal; the major space requirement is for sample preparation and analytical chemistry (21 CFR 58.43).

2. Facilities for Handling Test and Control Articles

If radiochemicals are used in these studies, proper provision for chemical storage, handling, and disposal is required and special safety precautions must be

taken for personnel (21 CFR 58.47). FDA recognizes that these aspects of a study are dealt with under the radiochemical licensing requirements for the testing facility.

This section also discusses the facilities for handling test and control articles, the necessity to prevent cross contamination or mixups in the test articles in use in the laboratory and the precautions that should be taken. This is generally accomplished through separation of functional areas (21 CFR 58.49) and the prudent precaution of working with only one test article at a time. It is clear that the test articles must be stored in a manner designed to preserve their integrity and identity. For most chemicals, this will be ambient or refrigerator storage.

D. Equipment

1. Equipment Design

The equipment to be used in pharmacokinetic studies must be of appropriate design and adequate capacity to function according to the protocol and shall be suitably located for operation, inspection, cleaning, and maintenance (21 CFR 58.61).

2. Maintenance and Calibration of Equipment

Although SOPs have not yet been mentioned as a section of the regulations, there is a clear requirement that SOPs must be in place covering all aspects of equipment maintenance and calibration (21 CFR 58.63). All operations must be documented.

My experience with this aspect of the regulations is that too much effort goes into calibration of routine equipment; companies should look carefully at contemporary equipment and the need (or lack thereof) for frequent calibration of measuring equipment. SOPs that were written years ago for this purpose may no longer be valid. Take as an example, balances and balance calibration. Contemporary electronic balances all have built-in calibration check systems; if the balance is not operating within specifications, an error message will be displayed and no further work will be accepted. Why then, do we find analytical laboratories where the SOP calls for calibrating such balances before every use and including check weight measurements as part of the calibration exercise? For the most part this cannot be justified technically and is a potential waste of resources.

Written records must be maintained for all inspections, calibrations, maintenance, and so on, for equipment used in a nonclinical laboratory study. This is generally done. What is often lacking is a set of written criteria for accepting back into service equipment taken out of service for routine or nonroutine maintenance or repair service. These specifications and records are required.

E. Testing Facility Operation

1. Standard Operating Procedures

The few sentences in Section 21 CFR 58.81, standard operating procedures, are likely to be the testing facility's major concern. SOPs are the basis of all documented procedures and results and, despite the overhead cost of establishing and maintaining SOPs, this must be done. This is probably where any testing laboratory will have to place its up front money in order to become compliant with the GLP regulations. Writing SOPs is expensive, time-consuming, and universally unloved. The requirement for SOPs is stated clearly as:

> A testing facility shall have standard operating procedures in writing setting forth nonclinical laboratory study methods that management is satisfied are adequate to insure the quality and integrity of the data generated (21 CFR 58.81[a]).

It is most important to note the language used here: *management* must be satisfied that this is a sound SOP that reflects current practice in the testing facility. If management is satisfied with the SOP, the opinion of anyone else is just that: opinion. Management shows its approval of the SOP through a signature on the SOP. What is not defined anywhere is, what is management? This is left up to the individual testing laboratory to determine and it will be found on that most basic SOP: how to write, approve, put into service, and revise SOPs. Of major concern in large testing laboratories is the need to make sure that only one version of an SOP is in circulation and use.

2. Reagents and Solutions

This two-sentence section of the regulations probably causes more heated discussion than any other part of the regulations since it requires that All reagents and solutions in the laboratory area shall be labeled to indicate identity, titer or concentration, storage requirements, and expiration date. Deteriorated or outdated reagents and solutions shall not be used (21 CFR 58.83).

The major objection lies in the expiration date requirement. Most testing facilities today adopt a system for expiration dates that is a combination of both professional expertise and practicality. Many reagents and solutions arrive at the testing facility with expiration dates assigned by the manufacturer. If so, use the material up to but not beyond the date printed on the bottle or the label. If there is no date printed, use a conservative approach and assign an expiration date of 35 years from the date the container is first opened. At the end of this time you should either dispose of the remaining material or, if professional judgment so allows, assign a new expiration date as a replacement for the date originally assigned.

3. Animal Care.

The section on animal care of the regulations (21 CFR 58.90) is not of major concern in pharmacokinetic studies. These regulations were written for the large toxicology testing facilities with hundreds of animals in use at any one time and the care of the test animals is most important. Pharmacokinetic studies, on the other hand, use very few animals both at any one time and in the aggregate; proper care and maintenance of these small numbers of animals over a relatively short experimental period are not difficult. This is not to say that animal care is to be ignored in these studies, only that minimal effort will be needed here to be in full compliance with the regulations, as opposed to other parts of the study, which will require larger expenditures of resources to remain in compliance.

Animal care in pharmacokinetic studies is generally limited to the raising or maintenance of the animals in the animal quarters and then transferring them to the pharmacokinetics laboratory for the short experimental period. This is opposed to the standard toxicology study, in which the animal quarters areas are used for long-term studies with many animals in each group. Care and maintenance of the test animals are vital to the long-term outcome of the studies.

F. Test and Control Articles

1. Test and Control Article Characterization

By the time a test article (chemical) enters pharmacokinetic studies, it has been characterized properly, impurities are known, and stability has been determined as required by the regulations. Analytical procedures have been developed and validated and the dosage form is understood (21 CFR 58.105).

2. Mixture of Articles with Carriers

The homogeneity, stability, and concentration of the active chemical have been determined prior to the start of pharmacokinetic studies (21 CFR 58.113). These sections of the regulations pertain primarily to standard nonclinical laboratory toxicological studies that are basic to understanding the health and safety aspects of the test article that is an actual or potential pharmaceutical entity. Ordinarily the active test article is mixed with a carrier for pharmacokinetic studies immediately before administration, and stability of the test article in the carrier is not an important concern.

G. Protocol for and Conduct of a Nonclinical Laboratory Study

1. Protocol

This section of the regulation (21 CFR 58.120) lays out the 12 required elements of a

study protocol. These should all be addressed by the study director and the test sponsor and represent the minimum requirements for protocol development.

The protocol should represent clearly the relationship between the present pharmacokinetic study with previously completed nonclinical laboratory toxicological studies. The relationship between species and the method of selecting doses for the pharmacokinetic studies should be made clear. The protocol is the study plan and identifies all the resources (personnel, space, and equipment) that will be required by the study. The protocol provides all the information needed for the master schedule and provides the framework for monitoring of the study by the QAU. The study is initiated when the protocol has been approved by the sponsor and signed by the study director.

The protocol may always be changed or revised (21 CFR 58.120[b]) but any such change must be documented, signed by the study director, dated, and maintained with the protocol. Ordinarily the study director also provides an opinion as to the effect, if any, of the protocol change on the outcome or interpretation of the study.

Nonadherence to the protocol or making undocumented changes to the protocol is one of the major sources of citations for noncompliance with FDA's GLP regulations. Such noncompliance has been documented over the years by the FDA and remains a significant problem. Most changes to the protocol are well intentioned and may even enhance the quality of the science and assist in the interpretation of the study data. This is, however, not the point. The point is that any change to the protocol must be documented; this is the general source of noncompliance: lack of documentation.

2. Conduct of a Nonclinical Laboratory Study

In this section are found the requirements for specific identification of specimens obtained during the study as well as the chain of custody needed to preclude error in recording and storage of data (21 CFR 58.130). The second part of this section, 21 CFR 58.130(e), contains the requirements for data recording and ownership. The requirements are quite simple and straightforward:

> All data generated during the conduct of a nonclinical laboratory study, except those generated by automated data collection systems, shall be recorded directly, promptly, and legibly in ink. All data entries shall be dated on the date of entry and signed or initialed by the person entering the data.

It would be difficult to express this any more clearly. There is no requirement for black ink, there is no requirement for the use of bound notebooks. These are institutionalized policies that we follow. Data pages are acceptable and, in many instances, preferable to the use of the standard single- or double-page notebooks. Patent or litigation concerns generally drive the requirement for recording data in bound notebooks and the use of black ink. Please note that the ownership of the data is shown by the dated signature of the person entering the data. The requirement for promptness means just that; the

observer of the

data being generated may not wait until tomorrow or next week to enter the data; this is not prompt. The data may not be recorded on one medium and then copied to another; this is not direct entry, it is transcription. The original entry is the raw data and must be retained. There is no requirement for witnessing the data or a second signature indicating review of the data; these are policies our individual companies have developed.

Computer raw data may be either the magnetic medium or the first signed and dated printout. Until we develop magnetic media that are more stable and software that will read all magnetic media, the written page remains the documentation of choice. We remain a long way from the paperless laboratory.

Data entries may be changed if needed but there are specific requirements for this; these requirements hold for either data on paper or data in a computer: Any change in entries shall be made so as not to obscure the original entry, shall indicate the reason for such change, and shall be dated and signed or identified at the time of the change.

This means that corrections can be made at any time in the future that an error is noted and a change is required. Corrections are to be made only by an individual with the authority to make the change.

H. Records and Reports

1. Reporting of Nonclinical Laboratory Study Results

Pharmacokinetic studies are reported, as are all other studies (21 CFR 58.185). The minimum content of a study report is given in this section and is a logical exposition of what was done, who did it, what were the results, and what is the interpretation of the results. If more than one scientist or other professional contributed to the study, those individuals are to be identified and their signed and dated reports are to be made part of the final report either by integration into the report or as an appendix to the report.

2. Storage and Retrieval of Records and Data

All raw data, documentation, protocols, final reports, and specimens generated as a result of a nonclinical laboratory study shall be retained (21 CFR 58.190). Exceptions are made for wet or fragile specimens or other specimens with a limited lifetime in storage.

In this section (21 CFR 58.190[b]) is found the requirement for a limited-access archives for the storage and expedient retrieval of all raw data; expedient has not been defined. Limited access means exactly that. The named archivist will have access, security ordinarily has access, but basically no other person has access if we are to maintain tight control over the archived data. Archiving does not necessarily mean that the data are

archived in their final and organized form. The data may be considered as archived when the study

director signs the final report and the data files pass from the control of study personnel to the control of the archivist. In many facilities the final inventory of the data, the final pagination and binding of the data, and the final copying of notebook pages, may take months but the data are archived once the study personnel no longer have free access to the data.

3. Retention of Records.

Retention of the records of a nonclinical laboratory study is set by the regulations (21 CFR 58.195) and the retention time depends on the type of application to the FDA and if the study was submitted in the first place. The retention period is 25 years, in general, from the time the application is approved or the results submitted but not acted upon. Records required to be retained may be retained either as original records or as true copies such as photocopies, microfilm, microfiche, or other accurate reproductions of the original records.

Contemporary technology is also available for storing all records on optical disc; this has great advantages in terms of space requirements as well as making duplicate copies for study as well as security. Optical storage, however, suffers from the same problems of obsolescence as do data that are computer- or magnetic medium-resident.

Quality Assurance is charged in this section with maintaining copies of the master schedule, protocols, and records of QA activities.

I. Facility Inspections for Regulatory Compliance

It is appropriate at this time to return to that part of the GLP regulations in which FDA asserts its right and intention to inspect facilities conducting nonclinical laboratory studies for compliance with these regulations (21 CFR 58.15). The procedures used by FDA's compliance inspectors (Consumer Safety Officers) are known and published [8]. Any inspection of a facility, regardless of the regulations involved, must be taken very seriously, since the consequences of findings of noncompliance may be extremely damaging to both testing facility and test sponsor. Compliance inspections may last from 2 days to 2 weeks or more, will cover all applicable regulatory concerns as described above, and will use continuing studies selected from the facility's master schedule. Completed studies may be audited for protocol adherence and data integrity. Standard operating procedures will be examined for accuracy. Animal care procedures will be monitored, as will be all aspects of analytical chemistry that support nonclinical laboratory studies.

The end result of a compliance inspection will be a narrative report that will be publicly

available under the Freedom of Information Act (FOI). Compliance inspections are becoming more thorough, inspectors are better trained, and the penalties for noncompliance are becoming ever more severe and costly. Today's FDA does not place great emphasis on the inspection of pharmacokinetic studies,

but with the increasing pressure from European and Japanese markets for conducting pharmacokinetic studies in compliance with the GLP regulations it is prudent to assume that tomorrow's FDA will look more closely at these studies.

IV. Summary

The Good Laboratory Practice (GLP) regulations as promulgated by the FDA have nothing to do with science and the design of pharmacokinetic studies. The GLP regulations have everything to do with the prudent management of studies and with the integrity of the data needed to reconstruct the study. Compliance with the regulations incurs costs for the testing laboratory, which are likely more than made up by working in an error-free facility where studies do not have to be repeated due to insufficient documentation or questionable practices.

Whether the scope of the GLP regulations includes pharmacokinetic studies is unclear. These studies are not included in the US and in Germany, for example, but are included in Japan and in the United Kingdom. The European Commission (EC) has not yet provided a definitive statement on this, but indications are that the EC is moving to place pharmacokinetic studies within the scope of the Member States' GLP regulations.

In this chapter we have explored the history of these regulations and have proceeded through an analysis of the major sections of the regulations. The lessons to be learned are that compliance with these good laboratory management practices is an investment wisely made by both sponsors and nonclinical laboratory testing facilities.

Note Added in Proof

The recent drive toward international harmonization of the technical requirements for the registration of pharmaceuticals has produced a change in the FDA's stated position on the regulatory requirements for toxicokinetic studies (9). The International Conference on Harmonization (ICH) is concerned with harmonization of technical requirements for the registration of pharmaceutical products among three regions: the European Union, Japan, and the United States. The FDA, in this *Federal Register* draft guideline notice, defines toxicokinetics as the generation of pharmacokinetic data in nonclinical toxicity studies or ancillary studies to assess exposure. These nonclinical studies are to supply information which, along with the toxicity findings, will contribute to developing additional nonclinical toxicity studies. This places toxicokinetic studies firmly into the nonclinical laboratory studies category, all of which are to be conducted according to the GLP regulations. However, while the FDA continues to consider pharmacokinetic studies as concomitant toxicokinetics which are integrated within the range of toxicity studies, the

new guidelines also

state that as a general principle, (It) should be noted that for those toxicity studies whose performance is subject to the Good Laboratory Practice (GLP) the concomitant toxicokinetics should also conform to GLP. Toxicokinetic studies retrospectively designed to generate specific sets of data under conditions which closely mimic those of the toxicity studies should also conform to GLP. The apparent interpretation must be that while the FDA does not consider pharmacokinetic studies as a requirement for product registration, the FDA also tells the pharmaceutical community that these studies are to be conducted in accord with the GLP regulations.

References

1. Title 21, *Code of Federal Regulations, Part 58.3(d).*

2. Fed. Reg. 41:225, 51207, 1976.

3. Title 40, *Code of Federal Regulations,* Part 792; Fed. Reg. 48:203, 53922, 1983.

4. Title 40, *Code of Federal Regulations,* Part 160; Fed. Reg. 48:203, 1983.

5. Title 40, *Code of Federal Regulations,* Parts 160 and 792; Fed. Reg. 54:158, 34034 and 34052, 1989.

6. Offic. J. Eur. Commu. L 147:1, 1975.

7. Offic. J. Eur. Commu. L 240:32, 1991.

8. Compliance Program Guidance Manual, Food and Drug Administration, Bioresearch Monitoring, Program 7348.808, Food and Drug Administration, Rockville, Maryland, February, 1992.

9. Federal Register, 59:40, 9755, 1994.

2
Methods to Assess Absorption in Drug Discovery

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I. Introduction

A preclinical pharmacokineticist, responsible for supporting drug discovery and development projects, has available various in vitro, in situ, and in vivo tools

and techniques to characterize the absorption of new chemical entities, to determine the rate and extent of absorption, to examine the effect of a new formulation on absorption, to investigate the site-specific absorption of a compound, or to determine the underlying mechanism(s) of drug transport or a drug-drug interaction. Since in vivo preclinical absorption, distribution, metabolism and excretion studies in animals are discussed in Chapter 12 of this book, this chapter will focus on the various in vitro and in situ methods available to assess drug absorption and transport in preclinical support studies, review the advantages and limitations of the technique, and, where possible, compare results obtained from the various techniques to in vivo findings. When selecting an in vitro or in situ drug absorption model, the following criteria should be considered:

Simplicity
Reproducibility
Rapid turnaround time
Predictability of in vivo absorption in humans

II. In Vitro Methods to Assess Drug Absorption

A. Cell Culture Models

Several human colon carcinoma cell lines, such as the Caco-2, HT-29, SW116, LS174T, and SW-480 cell lines, have been proposed as tools to investigate drug absorption [1,2]. The cultured epithelial cells undergo enterocytelike differentiation in culture and spontaneously differentiate into polarized columnar cells that are representative of the small intestine, with developed microvilli and polarized distribution of brush border enzymes. When grown on plastic membranes, epithelial cells result in a confluent monolayer and therefore serve as a model to study drug absorption [3,4]. To date, the Caco-2 cell line has been the most thoroughly investigated in vitro model and will be discussed in this section. The properties of Caco-2 cells comparable to those of small intestine absorptive cells include ion transport [3,4], vitamin uptake [5], and similar general morphological characteristics [6,7].

In vitro cell culture models have been utilized to assess the permeability and metabolism of drugs, to elucidate molecular mechanism(s) of drug transport, to provide information on pathways of drug degradation, and to explore the influence of structure on the absorption of new chemical entities [8,9]. Drug transport and absorption studies that have utilized the Caco-2 in vitro cell absorption model are summarized in Table 1. Advantages of the in vitro cell models include a rapid turnaround of information, the potential to decrease the number of animals studies, and the ability to utilize human tissue in transport and absorption studies.

Table 1 Application of Caco-2 Cell Model to Drug Absorption and Transport Studies

Application	Example	Reference
Examine factors affecting the transport of drugs	Investigate the effect of pH, glucose, temperature, inhibitors on the transport of a-methyldopa	16
	Examine the effect of mannitol concentration on transport	17
	Investigate the effect of temperature on various transport mechanisms	18
	Examine the influence of peptide structure on transport across the epithelial cell; explore the relationship of permeability coefficient across the Caco-2 monolayer and the number of potential hydrogen-bonding sites in the solute	10
Study drug transport mediated by various carriers	Characterize vitamin D receptor	18
	Identify bile acid carrier	19
	Investigate the transporter-mediated mechanism for glucose	20
	Examine the role of g-glutamyl transpeptidase in amino acid transport	21
	Explore the carrier-mediated transport for large neutral amino acids	
	Phenylalanine	22
	Proline	23
	Identify a dipeptide cotransport system involved in the efflux of cephalosporins across the basolateral membrane	24
Investigate metabolism reactions	Phase II metabolism	
	Sulfation of p-nitrophenol and dopamine	25
	Glucuronidation of p-nitrophenol	26
	Investigate vitamin A metabolism	27
	Study the metabolism of oxygenated derivatives of arachidonic acid	28
	Investigate the activity of acyl coenzyme A cholesterol	

activity in the presence of PD 128042 29
Investigate fatty acid uptake and metabolism 30

Several investigators have compared the results obtained from the Caco-2 cell model studies to in vitro, in situ, and in vivo observations in animals and humans. Conradi and co-workers compared the permeability coefficients calculated for selected peptides across Caco-2 monolayers to the permeability coefficients calculated from an in vitro rabbit model [10]. The permeability coefficients obtained in the Caco-2 cell model were qualitatively similar to the rabbit intestinal model. Another study compared the rate of epithelial transport of b-blocking agents in a Caco-2 absorption model to the rate of ileal absorption of these agents in a rat in situ model [11]. After results were normalized for surface area, the transport rates of four of five compounds were similar in the two models; however, there was no correlation between the in vitro permeability coefficients and the in situ data. Other investigators have examined the in vitro permeability coefficients of the nine compounds in the Caco-2 cell model and compared the findings to the permeability coefficients obtained in diffusion cells using intestinal segments (duodenum, jejunum, ileum and colon) of monkey, dog, and rabbit [12]. No correlations were found between the Caco-2 cell model and the duodenum, jejunum, and ileum segments of any species or the colonic segments of the dog and monkey. A linear relationship with a good correlation was obtained between the rabbit colon permeability and the Caco-2 permeability coefficient; however, the permeability obtained with the Caco-2 cell monolayer was about twice as high as that seen with rabbit colon tissue mounted in the diffusion chambers. This observation may be due to the lack of goblet cells and mucin in the Caco-2 cell monolayers. As more correlations are established between the Caco-2 absorption model and the various other techniques available to study drug absorption in animals, the Caco-2 absorption model may serve as a valuable screening tool in pharmacokinetic preclinical discovery support groups, thereby reducing in vivo animal testing.

Limited information has been published on the correlation between oral drug absorption in humans and the apparent drug permeability coefficients obtained from the Caco-2 cell model. A good correlation was obtained between historical data on oral absorption in humans and the permeability coefficients from the Caco-2 model for 20 xenobiotics or peptides with different structural properties, as shown in Figure 1 [13]. Permeability coefficients were derived using a Caco-2 stagnant fluid system model. The results indicated that the permeability coefficients of compounds that were absorbed in humans (>1% but less than 100%) or poorly absorbed (<1%) were 0.11×10^{-6} cm/s and $<10^{-7}$ cm/s, respectively. A similar correlation with between the permeability coefficients obtained from a stirred Caco-2 cell model and in vivo absorption data has also been reported [12]. These examples suggest that the Caco-2 in vitro absorption model may be useful in predicting the extent of absorption of a new chemical entity in humans.

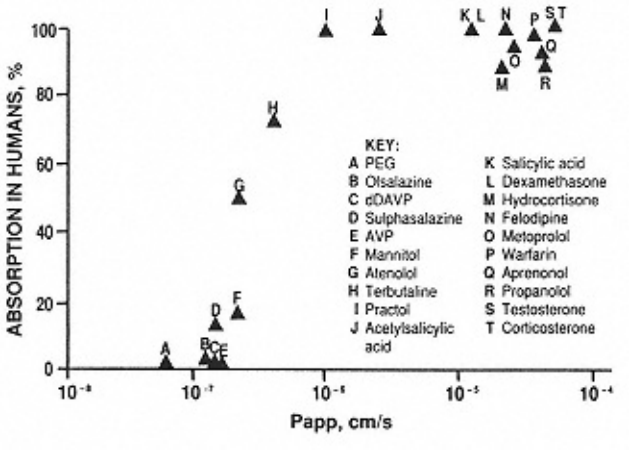

Figure 1
Absorption in humans following oral administration as a function of apparent permeability coefficient (Papp, cm/s) in the Caco-2 cell model. (Adapted from Ref. 13.)

Although many of the advantages of the Caco-2 cell model have been presented, the limitations of the model must be considered. From a practical standpoint, maintaining the cell line requires a designated aseptic area, training in aseptic techniques, and continuous maintenance of the cell line. From a physiological point of view:

1. The tissue in the villus contains more than one cell type.

2. The Caco-2 cell lines does not produce the mucus observed in the small intestine.

3. No P-450 drug-metabolizing enzyme activity has been reported in the Caco-2 cell line.

Experimental factors such as the permeable support membrane and the thickness of the unstirred water layer have also been reported to influence the interpretation of the Caco-2 cell studies [14,15].

Although several limitations of the cell culture model have been identified, the in vitro cell culture model has been extremely useful to investigate factors that affect drug transport and to understand carrier-mediated absorption in the intestine. More data regarding the correlation between in vivo absorption and in vitro permeability coefficients obtained from the Caco-2 cell model are required to validate the predictability of this absorption model.

B. Isolated Mucosal Cells

Procedures for isolating mucosal cells include mechanical agitation, scraping, hydrolytic collagenase enzymes, or chelating agents such as ethelynediaminotetraacetate (EDTA) [31,32]. Such isolated cell suspensions have been used to study enzyme activity, drug transport, and cellular metabolism. However, it must be noted that the isolated mucosal cells lack a submucosa and muscle layer that may present a diffusional barrier to transfer absorbed substances.

One study compared the intraduodenal bioavailability of theophylline, salicylate, a-methyldopa, and cefoxitin in rats to the mucosal uptake of the compounds. The relative order of drug absorption in the mucosal cells did not reflect the in vivo results [33].

The use of mucosal cells in drug absorption and transport studies is limited due to rapid autolysis. Another disadvantage of mucosal cells is that the isolation process requires opening the tight junctions, which destroys cell polarity, making it impossible to determine the specific membrane domain involved in cellular uptake. The method of isolating the mucosal cell may also have an impact on cell viability. One study compared the cell populations prepared from Weiser's EDTA intestinal perfusion [31] with Hartmann and co-workers' intravascular collagenase perfusion [32]. Although the enzymatic and metabolic activities of the two cell populations were comparable, cell viability determined by trypan blue dye exclusion test, ultrastructural appearance, and glucose uptake was similar to the in vivo values for cells isolated by the EDTA method [33]. Also, if separation is incomplete, the epithelial cell will be contaminated with other cell types. Given the limitations, the mucosal cell model falls short of meeting the selection criteria of a model to study or predict drug absorption.

C. Brush Border Membrane Vesicles.

There have been numerous methods reported to isolate brush border membrane vesicles (BBMV). These include disruption of the cellular structure followed by density centrifugation, immunoabsorbant chromatography, or precipitation of the nonbrush border enzymes with calcium or magnesium [3436]. The calcium or magnesium precipitation method seems to be the most widely used.

Brush border membrane vesicles have been used extensively to study mucosal uptake process, especially to investigate factors that influence mucosal uptake without interference of intracellular metabolism. Table 2 lists examples of BBMV applications. Other advantages of using BBMV preparations include [37]:

1. The ability to investigate the luminal and contraluminal sides separately.

2. Studying different compositions of the intra- and extravascular spaces.
3. The unstirred layer is small compared to tissue preparations.
4. Paracellular mechanism(s) do not contribute to transport.

Table 2 Application of Brush Border Membrane Vesicles to Drug Absorption and Transport Studies

Application	Example	Reference
Investigate transport	Study transport of bicarbonate, biotin, and riboflavin	3739
Study transport systems	Identify intestinal peptide transport system	40
	Examine the specificity of peptide transport	41
	Characterize Na+-dependent bile acid transport system	42
Examine factors affecting transport	Role of insulin in glucose absorption	43
	Effect of 3-azido-3' deoxythymidine on organic ion transport	44
	Effect of 1,25 dihydroxy vitamin D-3 deficiency on Ca+2 transport	45
	Investigate the effect of renal failure on biotin transport	46
	Examine age-related modifications of leucine uptake and D-glucose transport	47
	Determine the effect of stereochemistry on uptake of b-lactam antibiotics	40

The outcome of the transport experiment depends on the purity of the preparation, accurate measurement of uptake rates, viability of the uptake system during the experiment, and complete equilibration of the solutes across the vesicles prior to the assay with other cell types [38].

BBMV preparations have several disadvantages that limit their application to the assessment of drug absorption. First, the isolation procedure may either damage the membrane or may be incomplete, resulting in contamination of the preparation with other membranes. Since the cells are homogenized, active transport cannot be investigated. BBMV have demonstrated variability and time-dependent changes that also limit their use in drug absorption and transport studies [33].

D. Everted Tissue Techniques

1. Everted Sac Technique

To prepare an everted sac, a small length of the intestine is excised, turned inside out, filled with a fluid, and ligated at both ends. The sac is immersed in an oxygenated

solution that contains drug. The fluid inside the sac is assayed for drug and the rate of drug transfer across the membrane provides an estimate of drug permeability. The everted sac technique is simple, inexpensive, elimi-

nates variations due to blood flow, and permits sampling from mucosal and serosal sides. Everted sacs have been used to investigate questions regarding intestinal metabolism, uptake rates, and the permeability in different regions of the intestine. Studies with salicylamide in everted sacs demonstrated that permeability constants determined from the in vitro experiments did not necessarily reflect the permeabilities of the in vivo tissues [50].

There are several disadvantages associated with the use of everted sacs, including alteration of the data by experimental variables such as method of oxygenation, serosal sampling, and fluid loss, or damaging the tissue while removing it [50]. Since the tissue is removed from the animal and no longer associated with the blood supply, there is also always the potential of obtaining unrealistic rate and extent of absorption.

2. Intestinal Rings

Intestinal rings are prepared by excising a portion of the intestine, everting it over a glass rod, and cutting it into rings approximately 3050 mg. The rings are then incubated in an oxygenated culture media that contains drug. At the end of the incubation, the tissues are extracted and unchanged drug is measured. Intestinal ring preparations can be used to measure the rate of uptake and accumulation of a drug from the intestines of various laboratory animals and is easy, inexpensive, and reproducible. The structural integrity of the mucosal barrier is preserved and retains a large number of differentiated properties.

A comparison of salicylate, cefoxitin, a-methyldopa, and theophylline in vivo intraduodenal drug absorption in rats to the absorption in the intestinal ring model demonstrated a good correlation in rank order of drug absorption [51]. In a preclinical support group, this technique maybe useful in lead candidate selections. If a lead compound has to be selected from a series of compounds with similar structure and equal pharmacological and toxicological profiles, the intestinal ring preparation would be an easy and quick tool to narrow the selection of compounds with favorable absorption properties.

The limitations of intestinal ring preparations include accidental exposure of highly permeable areas of damaged tissue to incubation media, the lack of subcellular metabolism, lack of intact transcellular transport, and potential tissue degradation [50].

E. Isolated Tissue Techniques

In the isolated tissue technique, the epithelium is mounted as a flat sheet between two chambers often referred to as a Ussing apparatus [52]. A modification of the Ussing apparatus by Grass and Sweetana has also been reported [53]. The solutions on each side

of the chamber are oxygenated and maintained at physiological temperature. The test drug and markers for volume fluctuation or tissue viability are placed in the chambers. The chambers can be stirred using a gas

lift of O2/CO2 (95%/5%) at a flow rate of 1520 ml/min. Samples can be obtained from the serosal and mucosal chambers to study diffusion and permeability. The technique permits control of the electrochemical potential across epithelium, allowing the luminal and basolateral membranes to be studied together. The various application of isolated tissue models are summarized in Table 3. Drug absorption and transport in human intestinal tissues have been examined in this model.

A study in the literature has examined the in vitro permeability of a series of both hydrophilic and lipophilic compounds, including methanol, mannitol, D-glucose, ganciclovir, naproxen, PEG 900, PEG 4000, hydrocortisone, and progesterone. Data were obtained from four segments (duodenum, jejunum, ileum, and colon) of the rabbit, monkey, and dog intestine using a side-by-side diffusion cell [54]. The colonic tissues of the monkey, dog, and rabbit showed similar permeabilities. The results of this study demonstrate that the intestinal permeabilities vary from species to species and the selection of the appropriate animal model to predict absorption in humans is essential.

Table 3 Applications of the Isolated Tissue Techniques to Drug Absorption and Transport Studies

Application	Example	Reference
Study site-specific absorption	Study the transport of putrescine in duodenum, jejunum, and ileum	55
	Regional permeability of different-sized dextrans in the intestine	56
Examine the effect of disease state on drug transport	Determine the effect of cystic fibrosis on chloride transport	57
	Effect of rotavirus enteritis on jejunal potassium transport	58
	Effect of allograft rejection on transport in presence and absence of FK-506 immunosuppressive therapy	59
Investigate mechanisms of transport	Determine the mechanism of intestinal magnesium transporta	60
Study intestinal metabolism	5-Fluorouracil, desogestrel, cyclosporine	6163
Examine paracellular transport	Caprate and Caprylate	64
	Examine the effect of protease inhibitors on paracellular transport of vasopressin analogs	65
Drug-drug interactions	Effect of amiloride and ouabain on short chain fatty acid transport	66

aHuman tissue was utilized in the study.

Experimental factors such as altered tissue thickness or the presence of intact muscle could influence the results, causing changes in flux. One also must be concerned with tissue viability.

III. In Situ Methods to Assess Drug Absorption

Regardless of the type of in situ model selected (e.g., closed loop, single pass, or recirculating), in situ absorption models permit the study of individual organ processes and site specific absorption. The tissue is maintained intact with blood flow to the organ. Samples of drug solution from in situ loop experiments can be obtained to measure drug disappearance and metabolite formation. Applications of the in situ loop model to drug absorption and transport studies are listed in Table 4.

A. Closed Loop Studies

Two types of in situ loop studies have been reported: an anesthesia recovery method and the method of Doluisio et al. [67]. To perform an anesthesia recovery closed loop study, ligatures are formed in an intestinal region (e.g., duodenum, jejunum, or ileum), drug solution is introduced into the intestinal loop, the abdominal area is sutured, and the animal is permitted to recover from the anesthesia. Multiple blood samples may be obtained to measure the appearance of the drug in the blood. At a specified time, the animal is sacrificed, the loop is excised, and the amount of unabsorbed drug is determined. In this technique, the effect of anesthesia on the absorption process is minimized. Limitations of the technique include volume fluctuations and the fact that only one data point can be obtained from each animal's luminal content.

Doluisio and co-workers reported a technique that permits multiple sampling of the luminal content [67]. In this technique, the animal is anesthetized and two cannulae are inserted into the small intestine. The drug solution is introduced into the cannulated segment and multiple samples are withdrawn from the luminal solution during the experimental period. This technique is simple and has been proven to yield absorption rates that are realistic compared to the in vivo estimates of absorption rates of drugs such as aminopyrine, phenobarbital, haloperidol, prochlorperazine, salicylcic acid, and sulfaethidole [67]. The disadvantages of the technique include the use of an anesthetic that may alter blood flow to the intestine and the potential for volume fluctuations in the loop.

B. Perfused Loop Studies

The perfused loop study design is more physiological than the closed loop design. The cannulation procedure is identical to the procedure detailed above.

Table 4 Applications of In Situ Models to Drug Transport and Absorption Studies

Application	Example	Reference
Effect of absorption enhancers	Effect of EDTA, sodium caprate, sodium glycocholate on the absorption of insulin	69
	Effect of bile on cyclosporin absorption	70
Effect of enzyme inhibitors on absorption	Effect of aprotinin on insulin absorption	69
Site-specific absorption	Cyclosporin, carbamazepine, octreotide, and imidapril	7073
Examine the effect of chemical modification	Study the intestinal absorption of thyrotropin-releasing hormone by chemical modification with lauric acid	74
Examine the effect of micelles	Examine permeability of acyclovir across the intestine in mixed micelles	75
Examine drug-induced mucosal damage on intestinal absorption and metabolism	Study salicylamide metabolism following indomethacin and 5-fluorouracil treatment	76,77
	Absorption of sulfanilamide following mitomycin-C treatment	78
Examine carrier-mediated transport	Baclofen, b-lactam antibiotics, fosfomycin	7981
Examine the site of stereo inversion	Metabolic inversion of r(-) ibuprofen	82
Drug-drug interactions	Effect of dinoprost on imipramine absorption	83
Stereospecific absorption	Meta- and para-aminobenzoic acid absorption	84
Effect of chelating agents on absorption	Effect of citric acid, tartaric acid, penicillamine on the absorption of mercuric chloride	85
Investigate intestinal metabolism	Deacetylation of N-acetyl-L-cysteine	86
	Glucuronidation and sulfation of acetaminophen	87
Effect of gastrointestinal maturation on absorption	Cefazolin and cephradine absorption in 13-week-old rats	88

The drug solution is perfused into the loop and the flow rate can be regulated. The single pass technique can be adapted to permit recycling of the drug solution through the

intestinal lumen. In addition, the venous blood supply can be collected to monitor the appearance of drug. Some investigators have departed from the normal physiological situation by perfusing the intestine segment at a rate greater than the rate of propulsion of the intestine. Despite the high flow rates, a correlation between the in situ permeability and the percentage absorbed in humans has been reported, as shown in Figure 2 [68].

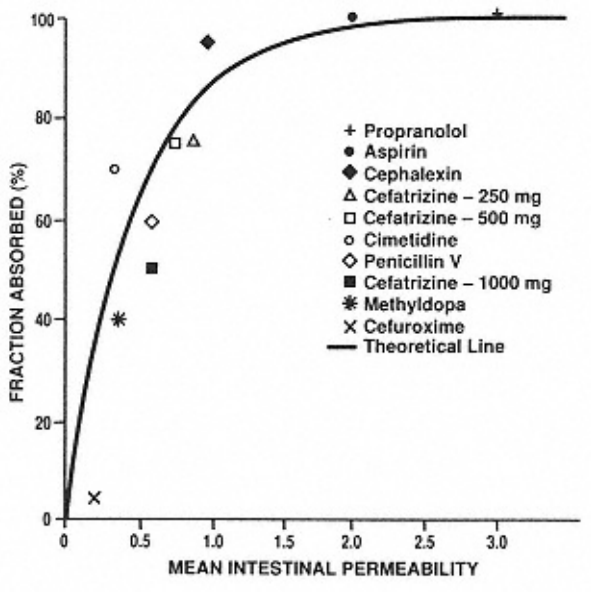

Figure 2
Fraction of dose absorbed as a function of mean dimensionless intestinal wall permeability. (From Ref. 68.)

C. Perfused Intestine-Liver Preparations

This technique uses a direct approach to examine the contribution of each organ/tissue in first pass metabolism and can be used to study the sequential processing of drug and metabolites by the intestine and liver [89]. A schematic illustration of the once-through vascularly perfused rat intestine-liver preparation is shown in Figure 3 [90]. The superior mesenteric artery and the hepatic artery are cannulated for the inflow of oxygenated perfusate to the intestine and liver, respectively. The bile duct can also be cannulated to permit full collection of bile. The portal vein is cannulated for sampling of venous perfusate from the intestine. In addition, the outflow from the hepatic vein can be sampled. Table 5 lists some of the applications of the model. The model is flexible and permits manipulation of the flow, intestinal site, concentration and perfusate.

IV. Conclusions

To extrapolate the findings from animal absorption and transport studies, an animal model that mimics human absorption must be selected. The limitations and assumption of the selected in vitro or in situ model cannot be overlooked.

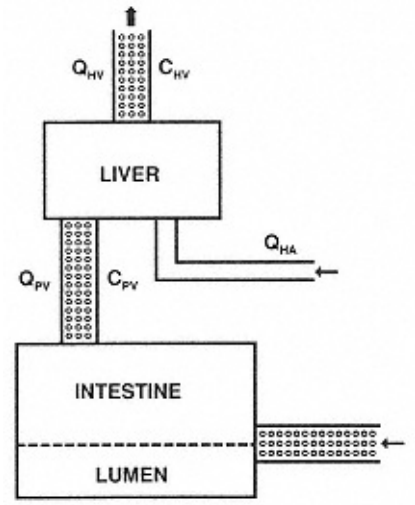

Figure 3
Schematic illustration of perfusion flow and perfusion apparatus in the once-through vascularly perfused intestine-liver preparation.(QHV, QPV, QHA, hepatic venous, hepatic portal and hepatic artery flows. CPV, CHV, steady-state drug concentration in the portal vein, and hepatic vein). (From Ref. 90.)

The selected technique to study drug absorption or transport should fulfill the defined selection criteria (e.g., simplicity, reproducibility, rapid turnaround time, and predictability of in vivo absorption in humans). Only a few articles have been published that correlate in vitro or in situ findings with human absorption. More correlations between in vivo absorption studies and the data generated

Table 5 Applications of Perfused Intestinal-Liver Preparations

Application	Example	Reference
Measuring extraction ratios	Determine the steady-state extraction ratio of enalapril in the intestine and liver	91
Contribution of intestine to first-pass metabolism	Intestinal metabolism of gentisamide and salicylamide regulates the availability of substrate for hepatic elimination	90,92
Investigate intestinal metabolism	The intestine is responsible for the formation of most of the 4-methylumbelerferone glucuronide	93

from in vitro and in situ models are necessary to validate extrapolations of the findings to predict absorption in humans.

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References

1. A. R. Hilgers, R. A. Conradi, and P. S. Burton. Pharm. Res. 7:902, 1990.

2. I. J. Hidalgo, T. J. Raub, and R. T. Borchardt. Gastroenterology 96:736, 1989.

3. E. Grassert, J. Bernareu, and M. Pinto. Am. J. Physiol. 248:C410, 1985.

4. I. Mohrmann, M. Mohrmann, J. Biber, and H. Muren. Am. J. Physiol. 250:C323, 1986.

5. G. Wilson, I. F. Hassan, C. J. Dix, I. Williamson, M. Mackey, S. Woodcock, and P. J. Artursonn. J. Control. Release 11:25, 1990.

6. R. Muthiah and B. Seetharam. J. Cell Biol. 105:235A, 1987.

7. M. Pinto, S. Robine-Leon, M. D. Appay, M. Kedinger, N. Triadou, E. Dussaeux, B. Lacroix, P. Simon-Assmann, K. Haffen, J. Fogh, and A. Zweibaum. Biol. Cell 47:323, 1983.

8. K. L. Audus, R. L. Bartel, I. J. Hidalgo, and R. T. Borchardt. Pharm. Res. 7:435, 1990.

9. G. Wilson. Eur. J. Drug Metab. Pharmacokinet. 15:159, 1990.

10. R. A. Conradi, A. R. Hilgers, N. F. H. Ho, and P. S. Burton. Pharm. Res. 8:1453, 1991.

11. P. Artursson. J. Pharm. Sci. 79:476, 1990.

12. W. Rubas, N. Jezyk, and G. M. Grass. Pharm. Res. 10:113, 1993.

13. P. Artursson and J. Karlsson. Biochem. Biophys. Res. Commun. 175:880, 1991.

14. P. Nicklin, B. Irwin, I. Hassan, I. Williamson, and M. Mackay. Int. J. Pharm. 83:197, 1992.

15. I. J. Hidalgo, K. M. Hillgren, G. M. Grass, and R. T. Borchardt. Pharm. Res. 8: 222, 1991.

16. M. Hu and R. T. Borchardt. Pharm. Res. 7:1313, 1990.

17. J. N. Cogburn, M. G. Donovan, and C. S. Schasteen. Pharm. Res. 8:210, 1991.

18. A. R. Giuliano, R. T. Franceschi, and R. J. Wood. Arch. Biochem. Biophys. 285: 261, 1991.

19. I. J. Hidalgo and R. T. Borchardt. Biochim. Biophys. Acta 1035:97, 1990.

20. A. Blais, P. Bissonnette, and A. Berteloot. J. Membr. Biol. 99:113, 1987.

21. T. K. Smith, C. L. Gibson, B. J. Howlin, and J. M. Pratt. Biochem. Biophys. Res. Commun. 178:1028, 1991.

22. I. J. Hidalog and R. T. Borchardt. Biochim. Biophys. Acta 1028:25, 1990.

23. P. L. Nicklin, W. J. Irwin, I. F. Hassan, and M. Mackay. Biochim. Biophys. Acta 1104:283, 1992.

24. K. Inui, M. Yamamoto, and H. Saito. J. Pharmacol. Exp. Ther. 261:195, 1992.

25. A. B. Kuzma, J. A. Garren, I. J. Hidalgo, and R. T. Borchardt. Life Sci. 49:1197, 1991.

26. S. Bjorge, K. L. Hamelehle, R. Homan, S. E. Rose, D. A. Turlock, and D. S. Wright. Pharm. Res. 8:1441, 1991.

27. T. C. Quick and D. E. Ong. Biochemistry 29:11116, 1990.

28. T. E. Riehl, J. Turk, and W. F. Stenson. J. Lipid Res. 33:323, 1992.

29. F. J. Field, E. Albright, and S. Mathur. Lipids 26:1, 1991.

30. R. J. Trotter and J. Stirch. J. Lipid Res. 32:293, 1991.

31. M. M. Weiser. J. Biol. Chem. 248:2536, 1973.

32. F. Hartmann, R. Owen, and D. M. Bissell. Am. J. Physiol. 242:G147, 1982.

33. I. Osiecka, P. A. Porter, R. T. Borchardt, J. A. Fix, and C. R. Gardner. Pharm. Res. 2:284, 1985.

34. A. Eicnholz, and R. K. Crane. J. Cell Biol. 26:687, 1965.

35. B. Steiger and H. Murer. Eur. J. Biochem. 135:95, 1983.

36. J. Carlson, K. Christiansen, and B. Bro. Biochim. Biophys. Acta 689:12, 1982.

37. S. A. Acra and F. K. Ghishan. J. Parenter. Enter. Nutr. 15:935, 1991.

38. D. D. Maenz, C. Chenu, F. Bellemare, and A. Berteloot. Biochim. Biophys. Acta 1069:250, 1991.

39. Y. Ikeda, M. Kitajima, and L. A. Turnberg. J. Clin. Gastroenterol. 14:S98, 1992.

40. H. M. Said, and I. Derweesh. Am. J. Physiol. 261:R94, 1991.

41. M. Friedrich, H. Murer, and E. G. Berger. Pflugers Arch. 418:393, 1991.

42. W. Kramer, F. Girbig, U. Gutjahr, S. Kowalewski, F. Adam, and W. Schiebler. Eur. J. Biochem. 204:923, 1992.

43. J. P. Bai and G. L. Amidon. Pharm. Res. 9:969, 1992.

44. W. Kramer, S. B. Nicol, F. Girbig, S. Kowalewski, and H. Fasold. Biochim. Biophys. Acta 1111:93, 1992.

45. J. M. Argiles, A. Zegri, J. Arbos, C. Garcia, and F. J. Lopez-Soriano. Int. J. Biochem. 24:631, 1992.

46. D. A. Griffiths, S. D. Hall, and P. P. Sokol. J. Pharmacol. Exp. Ther. 260:128, 1992.

47. R. Kaune, I. Kassianoff, B. Schroder, and J. Harmeyer. Biochim. Biophys. Acta 1109:187, 1992.

48. H. M. Said, N. D. Vaziri, F. Oveisi, and S. Hussienzadham. J. Lab. Clin. Med. 120: 471, 1992.

49. V. F. Sacchi and S. Magagnin. Mech. Ageing Dev. 63:257, 1992.

50. W. H. Barr and S. Riegelman. J. Pharm. Sci. 59:154, 1970.

51. P. A. Porter, I. Osiecka, R. T. Borchardt, J. A. Fix, L. Frost, and C. Gardner. Pharm. Res. 2:293, 1985.

52. H. H. Ussing and K. Zerahn. Acta Physiol. Scand. 23:110, 1951.

53. G. M. Grass and S. A. Sweetna. Pharm. Res. 5:372, 1988.

54. N. Jezyk, W. Rubas, and G. M. Grass. Pharm. Res. 9:1580, 1992.

55. A. M. Dumontier, P. Brachet, J. F. Huneau, and D. Tome. Pflugers Arch. 420:329, 1992.

56. N. Pantzard, B. R. Westrom, A. Luts, and S. Lundin. Scand. J. Gastroenterol. 28: 205, 1993.

57. E. V. O'Loughlin, D. M. Hunt, K. J. Gaskin, D. Stiel, I. M. Bruzuszcak, H. C. Martin, C. Bambach, and R. Smith. Am. J. Physiol. 260:G758, 1991.

58. J. P. Woodard, W. Chen, E. O. Keku, S. C. Liu, J. G. Lecce, and J. M. Rhoads. Am. J. Physiol. 265:G388, 1993.

59. T. K. Lee, M. A. Cardona, A. G. Kurkchubasche, S. D. Smith, A. R. Muellar, K. K. Lee, M. I. Rowe, and W. H. Schraut. J. Surg. Res. 52:605, 1992.

60. L. L. Hardwick, M. R. Jones, N. Brautbar, and D. B. Lee. Miner. Electrolyte Metab. 16:174, 1990.

61. P. Smith, C. Mirabelli, J. Fondacaro, F. Ryan, and J. Dent. Pharm. Res. 5:598, 1988.

62. S. Madden, D. J. Back, C. A. Martin, and M. L'E. Orme. Br. J. Clin. Pharm. 27:295, 1989.

63. J. F. Tjia, I. R. Webber, and D. J. Back. Br. J. Clin. Pharmacol. 31:344, 1991.

64. T. Sawada, T. Ogawa, M. Tomita, M. Hayashi, and S. Awazu. Pharm. Res. 8:1365, 1991.

65. A. L. Ungell, A. Andreasson, K. Lundin, and L. Utter. J. Pharm. Sci. 81:640, 1992.

66. W. von-Engelhardt, M. Burmester, K. Hansen, G. Becker, and G. Rechkemmer. J. Physiol. Lond. 460:455, 1993.

67. T. Doluiso, N. F. Billups, L. W. Dittert, E. T. Sugita, and J. V. Swintosky. J. Pharm. Sci. 58:1196, 1969.

68. G. L. Amidon, P. J. Sinko, and D. Fleischer. Pharm. Res. 5:651, 1988.

69. M. Morishita, I. Morishita, K. Takayama, Y. Machida, and T. Nagai. Biol. Pharm. Bull. 16:68, 1993.

70. Y. Cakaloglu, G. Marinos, J. Marsden, T. J. Peters, R. Williams, and J. M. Tredger. Clin. Sci. 84:675, 1993.

71. L. E. Riad and R. J. Sawchuk. Pharm. Res. 8:1050, 1991.

72. G. Fricker, J. Drewe, J. Vonderscher, T. Kissel, and C. Beglinger. Br. J. Pharmacol. 105:783, 1992.

73. P. Jeffrey, G. T. Tucker, A. Bye, H. K. Crewe, and P. A. Wright. J. Pharm. Pharmacol. 43:715, 1991.

74. Y. Yamada, M. Endo, M. Kohno, M. Otsuka, and O. Takait. Arzneimittelforschung. 42:457, 1992.

75. K. Yamada, M. Murakami, A. Yamamoto, K. Takada, and S. Muranishi. J. Pharm.

Pharmacol. 44:717, 1992.

76. G. B. Park, Z. Shao, and A. K. Mitra. Pharm. Res. 9:1262, 1992.

77. J. Nakamura, M. Katayama, K. Nishida, and H. Sasaki. Chem. Pharm. Bull. 40:1261, 1992.

78. J. Nakamura, M. Katayama, K. Nishida, and H. Sasaki. Chem. Pharm. Bull. 40:1011, 1992.

79. M. Mizuno, S. Kawabata, T. Hamaura, M. Hasida, and H. Sezaki. Toxicol. Appl. Pharmacol. 97:415, 1989.

80. A. Polache, J. M. Pla-Delfina, and M. Merino. Biopharm. Drug Dispos. 12:647, 1991.

81. P. J. Sinko and G. L. Amidon. J. Pharm. Sci. 78:723, 1989.

82. T. Ishizawa, M. Hayashi, and S. Awazu. J. Pharmacobiodyn. 14:82, 1991.

83. K. Arimori, M. Deshimaru, and M. Nakano, J. Pharm. Pharmcol. 44:15, 1992.

84. A. Yamamoto, T. Sakane, M. Shibukawa, M. Hahida, and H. Sezaki. J. Pharm. Sci. 80:1067, 1991.

85. T. Endo, S. Nakaya, and R. Kimura. Pharmacol. Toxicol. 68:171, 1991.

86. K. Sjodin, E. Nilsson, A. Hallberg, and A. Tunek. Biochem. Pharmacol. 38:3981, 1989.

87. D. Goon and C. D. Klaassen. J. Pharmacol. Exp. Ther. 252:201, 1990.

88. E. Morita, N. Mizuno, M. Nishikata, and K. Takahashi. J. Pharm. Sci. 81:337, 1992.

89. H. Hirayama, X. Xu, and K. S. Pang. Am. J. Physiol. 20:G249, 1989.

90. X. Xu, H. Hirayama, and K. S. Pang. Drug. Metab. Dispos. 17:556, 1989.

91. K. S. Pang, W. F. Cherry, and E. H. Ulm. J. Pharmacol. Exp. Ther. 233:788, 1985.

92. H. Hirayama and K. S. Pang. Drug. Metab. Dispos. 18:580, 1990.

93. C. L. Zimmerman, S. Ratna, E. Leboeuf, and K. S. Pang. J. Chromatogr. 563:83, 1991.

3
Drug Delivery Systems: Effects of Pharmacokinetics on Design, Evaluation, and Production

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I. Introduction

This chapter has not been written to be a how to primer for the design, evaluation, and production of drug delivery systems. The author certainly does

not claim that this chapter is an authoritative and comprehensive treatise on formulation. Such a scope would require a weighty volume rather than a short essay. The objective of this chapter is to discuss the interaction that has occurred in the last 20 years or so between the old, established art and science of formulation and the scientific adolescent we know as pharmacokinetics. The thesis advanced in this apologia is that pharmacokinetics has had, and will continue to have, a radical (even revolutionary) effect on the approaches now used in Western Europe and North America in the design, development, and production of drug products.

In 1963, formulators used a mixture of empiricism intuition and knowledge of the chemistry of drugs. In 1993 formulators use much less intuition and rely not just on chemical data pertaining to drugs but also on *biological* data. More specifically, they exploit our knowledge of pharmacokinetics in order to produce drug delivery systems that can reasonably be expected to perform within the body in an appropriate and reliable manner.

Pharmacokinetics has forced formulators to extend their professional horizons from the patient's lips to the patient's kidneys.

Those of us who were involved in the development and production of drug delivery systems in the 1960s will recall the exhilarating effect the then-new discoveries of pharmacokinetics and biopharmaceutics had on formulation. At last we began to appreciate the true potential of drug delivery design. However, the initial effects of experimental data generated by biopharmaceutics and pharmacokinetics were by no means universally positive. With depressing frequency, pharmaceutical scientists in the 1960s and 1970s reported data that clearly demonstrated that although different brands of the same product might meet fully with all the then standards of United States Pharmacopoeia (USP), their performance in vivo was significantly different. In some cases the differences in blood levels were such that therapeutic equivalence between the different brands was highly improbable. As a result of these findings, USP introduced dissolution tests. Also, regulatory agencies, such as the Health Protection Branch (HPB) in Canada, and Food and Drug Administration (FDA) in the United States became increasingly aware that biological availability was an important attribute for many drug delivery systems and must therefore be quantified in the documentation required for product approval. I suppose these developments might well be regarded as somewhat negative, since they required us to remedy previously unperceived deficiencies in our formulation or production methods.

However, the majority of the effects of pharmacokinetics on the design, evaluation, and production of drug delivery systems have clearly been positive. Pharmacokinetics has

added an extra dimension to the pharmaceutical formulator's vision, allowing new and more efficient approaches to their work. This chapter outlies some of the areas where the effect of pharmacokinetics has been most noteworthy.

II. Effects of Pharmacokinetics

A. Preformulation

Preformulation is probably the most obvious effect that pharmacokinetics has had on the art and science of formulation. It is difficult to determine with any certainty when the practice became common. However, we can state that whereas it was unusual before 1960, it had become the norm in most research-based pharmaceutical companies by 1990. In some companies, preformulation studies are conducted by those who are also involved in the design and evaluation of drug delivery systems; in others, specialist groups have been formed that deal exclusively with preformulation work.

Preformulation can be defined as a reconnaissance in depth to define the properties of a drug molecule likely to affect the design and performance of the drug delivery system. Although preformulation data do not necessarily tell us which path to follow in formulation work, it very often does inform us which paths are dead-ends. Preformulation is a good example of the successful philosophy that prevention is better than cure. Originally, preformulation was largely concerned with how possible incompatibilities between drugs and excipients might affect the stability of drug formulation. However, when, during the 1960s, an increasing body of experimental data clearly demonstrated the deficiencies in vivo of then commercialized drug products, the biopharmaceutical aspects of preformulation became increasingly important.

There is no standard menu accepted by all pharmaceutical companies for the activities of a preformulation group. The following list summarizes areas of study common to many such groups:

1. pK
2. Particle size spectrum
3. Aqueous solubility
4. pH solubility profile
5. Dissolution rate of pure drug substance
6. Distribution coefficient or some measure of drug membrane flux
7. Tests for polymorphism
8. Scan of incompatibilities with excipients
9. Stability scan

Of the tests listed above, the last two obviously pertain to stability, while the first seven clearly have substantial pharmacokinetic implications and, in particular, have considerable implications for the absorption process. The importance attached, and the amount of effort assigned, to the stability aspects of preformulation has in many companies tended to decrease, at least relatively, in recent

years. Thus, for example, Monkhouse [1,2] has argued articulately and powerfully that some types of preformulation tests for incompatibilities are academic in the worst sense of the word. He has indicated that studies in which powder mixtures of, say, 50% drug and 50% excipient exposed to storage at elevated temperature may well show interactions, but whether or not such interactions are of practical importance may well be debatable. In the finished dosage form, the excipient may only be present at the 1% level and the drug at 1%. Under such conditions, the diluting effect of all the other components in the formulation may well prevent the incompatibility from ever having any practical effect. As a result of these criticisms and, in some cases, after examination of their own experimental data, certain companies in the pharmaceutical industry have recently reduced the scope of their incompatibility testing. However, stability studies of the pure drug substance remain an essential part of preformulation studies, and FDA will expect to see data of this type in new drug applications (NDAs).

There are two prerequisites for a preformulation study: availability of drug in reasonable quantities, and a validated assay method. Since in some companies there has been a tendency to push preformulation earlier in the overall research and development sequence, the availability of drug substance can present a real problem. Considerable ingenuity has been shown by some preformulation groups in miniaturizing preformulation tests so that demands for drug substance are reduced. However, the availability of reasonable quantities of drug for preformulation studies is sometimes still a source of friction between the synthesis group and those involved in preformulation. There is a danger that if preformulation is undertaken at too early a stage in the research and development process, the synthetic method used for the production of drug substance may change, and this may require that some preformulation tests (e.g., stability or particle size) have to be repeated.

The development of stability-indicating assays for drug substances with molecular weights of less than about 500 Daltons (i.e., micromolecules), has become almost routine in many pharmaceutical companies. However, for macromolecular drug substances the question of the type and number of analytical tests needed in order to define adequately the nature of the drug substance is still a somewhat vexed question. Indeed, the whole question of how to conduct preformulation studies for macromolecules is still in a state of flux.

For drugs with basic or acidic functional groups, the determination of pK is a common preformulation exercise. The extent of drug ionization at prevailing gastrointestinal pH can be, in accordance with Brodie's theories, a critical factor in defining the extent and location within the gastrointestinal tract where orally administered drug is likely to be

absorbed. Until quite recently, many formulation drugs dealt very largely with micromolecular drugs that were weak electrolytes. However, it is noteworthy that a number of classes of potential new

drugs, such as some of those being examined by the National Cancer Institute (NCI) are quite strong electrolytes. For these compounds dissolution is unlikely to present a problem, but passive transport across biological membranes may be the rate-limiting step in the absorption process.

It has been well recognized for many years that for drugs of low intrinsic aqueous solubility, the particle size of the drug substances can, under some conditions, play an important role in the control of dissolution rate. There is, as of the spring of 1994, an increasing belief that our specifications for particle size for both drugs and excipients have in the past not always been sufficient. Thus, the USP Subcommittee on Excipients is presently investigating the development of more extensive particle size specifications for excipients. It seems likely that preformulation studies for new drug substances may, in the future, be concerned with defining the whole particle size spectrum rather than just the modal or median value.

Measurement of aqueous solubility and pH solubility profile has obvious relevance to absorption from the gastrointestinal tract; however, it should be appreciated that these equilibrium (classic thermodynamic) values may be of less importance than determination of dissolution rate (kinetic). Many preformulation groups use equipment such as the Wood apparatus to determine the intrinsic dissolution rate of the pure drug substance. The suggestion has been made that if the intrinsic dissolution rate constant is greater than 1 mg min^{-1} cm^2, the drug is unlikely to exhibit dissolution-related absorption problems. However, if the values is less than 0.1, such problems are likely and thus the oral route of administration should probably be avoided [3]. This empirical rule must obviously be modified for drugs of very low or very high dosage.

Measurement of an oil/water partition coefficient is a common segment of many preformulation programs. It appears that this value is determined because of the implicit or explicit assumption that it is predictive of passive drug membrane flux. Although there may be such a relationship within members of the same chemical class of drugs, the universality of the value of such predictions is very doubtful. Apart from the absence of any truly substantial body of experimental data that would allow induction of such a general relationship, there are serious theoretical objections to this theory. First, the distribution coefficient is an equilibrium (classic thermodynamic) value, whereas drug membrane flux is a kinetic term. Second, whereas biological membranes show a considerable degree of structure, a hydrocarbon, alcohol, or fixed-oil bulk phase has very little structure. Thus, one would not expect a universal correlation between partition coefficients and drug membrane flux values. It is not surprising, therefore, that preformulators in many companies now place increasing emphasis on drug membrane

flux values determined from use of isolated animal tissues. For example, the everted sac technique is quite commonly used [4]. It will be appreciated that since we are using dying animal tissue in

such tests, the correlation of the data we may obtain with that observed in the living, intact human may not be perfect. However, the general consensus is that such tests are often at least semiquantitative in their predictive power.

Since it is well established that polymorphism* is common for many drug classes, preformulation tests designed to elucidate whether the drug substance can exist in more than one crystal habit are standard, and such techniques as infrared spectroscopy and x-ray crystallography are widely used in such studies. Since different polymorphs may be characterized by substantial differences in dissolution rate, these determinations are of obvious importance with respect to oral drug absorption.

Preformulation activities in some companies now encompass some animal experimentation designed, for example, to determine absolute bioavailability by comparing the blood level/time profile for the orally administered solution or suspension with that of an intravenous dose. Such data can be of value in revealing a possible first-pass effect. However, since both route and extent of drug metabolism can vary substantially between species, this approach has obvious limitations. Perhaps more attractive is the possible use of cell cultures of human hepatocytes as tools for evaluating drug metabolism.

It is apparent that a major part of present-day preformulation studies has been strongly influenced by pharmacokinetics, rather than simply chemical considerations. This trends is likely to continue. One new area that may become of increasing importance to preformulation is the definition of the influence of chirality on the properties of new chemical entities. During the past decade or so, pharmaceutical scientists have been giving increasing attention to the effect of chirality in both pharmacodynamics and pharmacokinetics. The work of such groups as those led by Briens, Beckett, and Campbell has illuminated this area of fundamental importance. Regulatory agencies, both in Europe and North America, are now requiring sponsors to provide comprehensive data on this topic. In addition, a number of manufacturers of drug substances now have available chirally specific methods of manufacturing highly pure isomers at economically acceptable prices. Chirality, in some instances at least, not only modifies pharmacokinetic and pharmacodynamic properties but also can require modification of the design and production of drug delivery systems. Thus, Romero and his co-workers have shown that, for ibuprofen, the differences in crystal habit between the R and S isomers have several substantial effects on formulation *per se* [57].

*Polymorphism, when used in pharmacokinetics, usually refers to the existence within the population of two or more distinct subgroups that show significant difference in pharmacokinetic behavior. In this chapter, the term is not used in this sense but refers to crystal structure.

B. Dissolution Testing

Dissolution testing is surely the offspring of pharmacokinetics; whether the parent would like to acknowledge the relationship is debatable. Before the advent of pharmacokinetics, pharmacopoeial tests gave very little attention to quantifying the rate of release of drug from delivery systems. As data was published showing that for several important drugs, marketed versions of the same drug product, although complying fully with all the applicable pharmacopoeial standards, showed very substantial variation in the blood concentrations they produced, the pressure to adopt some test to quantify drug release became very strong.

Since the majority of drugs that pharmaceutical scientists were developing in the period from about 1960 to 1980 were weak electrolyte micromolecules, it was not unreasonable to assume that dissolution was the rate-determining step in the overall sequence of disintegration, dissolution, membrane flux, and intracorporeal transport to the general circulation. Thus, there were high hopes that dissolution testing would not only be of use as a quality control test to monitor batch-to-batch variability but might also prove to be quantitatively predictive of blood levels that any given drug delivery system might produce. The topic of in vitro/in vivo correlation is considered in Section D of this chapter. For many drugs the correlation between dissolution test results and in vivo performance remains obscure. Dissolution tests retain value for controlling batch-to-batch variability and are used by some workers in the development of new formulations. USP presently contains several types of dissolution test equipment used for various drug delivery systems. Considerable ingenuity has been exhibited in the design of dissolution test equipment, but at present the cynic might argue that the major beneficiaries have been those who manufacture such equipment. We hope that the situation may improve radically in the next decade.

USP now includes dissolution tests for practically all conventional oral drug delivery systems. The USP Bioavailability and Dissolution Subcommittee is presently wrestling with the thorny topic of developing dissolution test specifications for modified release drug delivery systems.

C. Bioavailability and Bioequivalence

One of the most striking differences between pharmaceutical formulation in the 1990s and practices common in the 1960s is the importance now given to the in vivo performance of drug delivery systems. Bioavailability considerations have a major, if not paramount, place in the objectives of many formulating projects. Of course, FDA and other regulatory agencies require bioavailability or bioequivalence data to be submitted

with NDAs or ANDAs, but in addition to data pertaining to products to be marketed, the sponsor will often conduct other biostudies at various stages during the formulation sequence.

Because of the present limitations in dissolution testing in terms of in vivo predictions, a number of formulation groups now use pilot scale biostudies at a relatively early stage of product development to check whether they are at least on the right track in terms of the bioavailability properties of their product. A study involving only four to eight subjects will have a relatively low statistical power, but the results of such studies are of direct relevance to performance in humans. Fine tuning of dissolution profiles, as is performed by some formulators may, on some occasions, be an academic exercise of little practical value.

Bioavailability studies are also important in building a bridge between a formulation used extensively in clinical testing and one selected to be used in the final market product. Regulatory agencies will quite properly require that bioequivalence be established between the formulation on which clinical justification for approval is based and that which will be marketed. During the past year or so, FDA has exhibited a propensity to give great weight to even quite nice differences between batches produced in research and development and those used in the production of market batches. It is therefore essential that those responsible for the production of the batch to be designated the production bioavailability batch be punctilious in ensuring that it meets all relevant FDA requirements. The agency normally requires that the batch must be made on the equipment to be used in the production of the market product, and the batch size shall be sufficiently large to be deemed representative of production conditions. Thus, for tablets the batch size should normally be at least 100,000 tablets.

Bioavailability tests (if they are to have an 80% power of detecting a 20% difference between two formulations, as is the normal requirement of a bioequivalency test) usually require at least 18 subjects. This makes such tests quite expensive. As of 1994 it is unusual to find that a full bioavailability study on, for example, 24 subjects will cost less than $60,000. One encouraging development in this area is the use of neural network computing techniques, which appear to have very considerable potential to increase the statistical power of biostudies that only use a small number of subjects [8,9]. If these hopes are realized, formulators are likely to make much more use of biostudies as part of formulation development they presently do.

D. In Vitro/In Vivo Correlations

Perhaps the most vexed question at the formulation pharmacokinetics interface is the present limited success in developing any relationship between in vitro test data (primarily dissolution results) and in vivo performance data, such as the area under the plasma concentration/time profile. We pharmaceutical scientists continue to search like medieval alchemists for the Philosopher's Stone that will solve our problems in this area. So far,

this rock appears to be remarkably well hidden!

Even the most exhaustive of studies of presently available data have found little to suggest any universally applicable relationship between in vitro test results and in vivo performance. For example, Kottke and her co-workers, who had access to extensive FDA files, were unable to find any substantial evidence of correlation, although a wide variety of possible mathematical relationships were explored [10] (see Table 1). In 1992, the Health Protection Branch of Canada, in association with FDA and USP, organized an International Open Conference on the topics of dissolution, bioavailability, and bioequivalence. A number of both the invited speakers and other participants discussed in vitro/in vivo correlations. In particular, the presentations by Leeson and by Welling, as well as the summary reports, provided some very useful information for those interested in exploring in detail this most fascinating, but often frustrating, topic [11].

It may be that the present lack of success in demonstrating any general relationship between in vitro and in vivo data derives primarily from limitations of the presently most widely used dissolution test equipment (the basket and paddle methods), both of which are essentially nonsink and do not readily lend themselves to smooth, controllable changes in pH. However, the flow-through cell apparatus, which recently became official in USP, may well offer the possibility of a breakthrough in this important field. At present, there is insufficient published data to define the full scope of the flow-through cell in this area. We hope that within the next few years we will be in a position to define, with precision, the exact utility of this equipment.

Another positive development that may lead to a resolution of the in vitro/in vivo problem is the use of deconvolution techniques, such as those based on statistical moment theory, to estimate directly the true in vivo dissolution time. Perhaps a wider application of these techniques will assist us in delineating a functional relationship between in vitro dissolution and in vivo performance.

If substantial progress is made in this area, we may confidently expect a reduction in the use of bioavailability studies as an aid in formulation development and an increase in the use of dissolution testing for this purpose. The advantages, in terms of both time and expense, make this possible development eminently desirable.

E. Process Validation

The Federal Register section on Good Manufacturing Practice states (211.110 (a)) inter alia:

> control procedures shall be established to monitor the output and to validate the performance of those manufacturing processes that may be responsible for causing variability in the characteristics of in-process material and the drug product.

Table 1 Number of In Vivo-In Vitro Correlations Found among the Total Number Investigated

Drug	Type of parameters evaluated	Number of ANDAs evaluated	Total number of correlations evaluated	Number of correlations found to exist
Diazepam	All	16	96	2
	Test/reference		72	3
Doxepin HCl	All	16	18	0
Ibuprofen	All	50	54	2
	Test/reference		54	3
Lorazepam	All	17	54	2
Total	Test/reference		54	6
		99	402	18

Source: Ref. 10.

It is noteworthy that tests that are assumed to have relevance to the pharmacokinetic attributes of the drug delivery system (dissolution and disintegration) figure prominently in process validation studies. Process validations, if conducted appropriately, should allow us to have a high degree of assurance that the process will reliably produce batches of pharmaceutical product that show little variability. We would then have good reason to believe that the release of drug into the biosphere will be essentially independent of batch and remain essentially reproducible over the extended period of time during which the product remains on the market. Figure 1 shows the steps required in a prospective validation sequence.

F. Optimization.

The process of formulation optimization allows the application of empirical mathematical techniques to determine the best compromise formulation or process for drug delivery systems [12]. Optimization replaces intuitive formulation approaches and trial-and-error methods; see Figures 2 and 3. Intuitive formu-

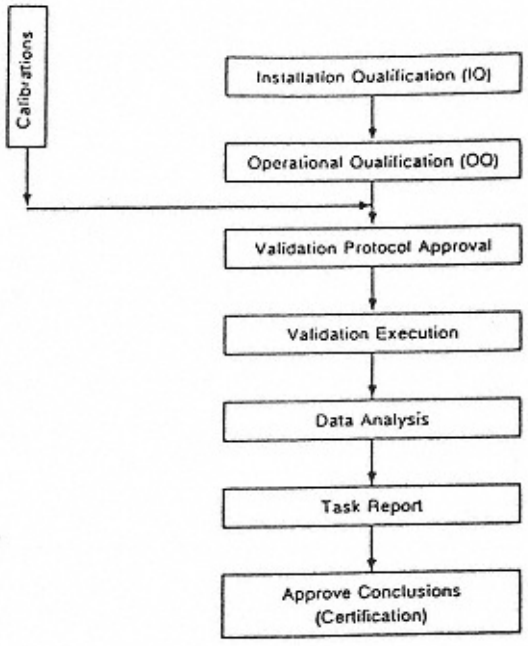

Figure 1
Prospective process validation. (From Lingnau. Drug Dev. Ind. Pharm. 15 (6 & 7):1040, 1989.)

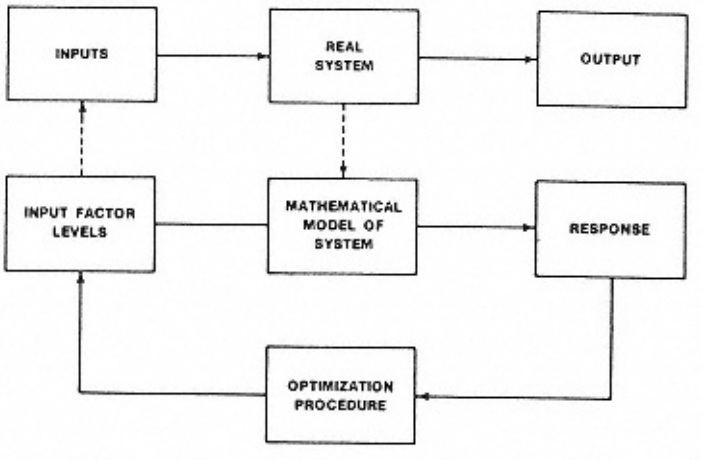

Figure 2
Flow chart for optimization. (From Schwartz. Modern Pharmaceuticals, 2nd Ed., p. 808, 1990.)

lation can be remarkably successful, but the number of practitioners of this art, which is based on a wide experience of dealing with many drug substances, is rapidly diminishing and probably in the future they will become increasingly rare. Trial-and-error methods of formulation are crude and, unless the number of possible formulation and processing variables being considered is very small, are most inefficient. Optimization techniques are flexible and allow the formulator to deal with a multivariate system with efficiency and a very high degree of success. Among the desirable dosage form attributes that can be included in the optimization program are those that impinge on pharmacokinetics. Thus, it is common to include dissolution time, and perhaps include in vivo response data such as blood levels or areas under the plasma concentration/time profile. It seems likely that these biological parameters will become increasingly important in pharmaceutical optimization studies and will allow pharmacokinetic considerations to permeate more thoroughly into the whole fabric of formulation.

G. New Drug Delivery Systems

The findings of pharmacokinetics have stimulated formulators to explore the potential of various routes of administration. Pharmaceutical scientists have shown considerable ingenuity in designing novel drug delivery systems that have the potential for improved therapeutic response, such as results if a first-pass effects is avoided or if the concentration of drug in the plasma is controlled, or if drug is preferentially delivered to the desired target organ.

Determination of
- Product profile
- Specifications for starting materials
- Test methods for starting materials and drug product
- Manufacturing process (draft)
- Critical steps

Optimization
- Process parameters
- Tolerance limits
- Preservation

Manufacturing process for clinical trials/validation development phase
- Validation protocol (prospective)
- Validation results (equipment, IPC, stability, quality testing)
- Validation report

Scaling up
- Equipment
- Process

Optimization

Manufacturing Process for Production
- Validation on the basis of results of the development phase
- Validation protocol (prospective)
- Validation data
- Calibration of measuring devices
- Qualification of (premises and) equipment
- Results of IPC
- Results of quality testing
- Validation report

Figure 3
Optimization and validation in development phase. (From Lingnau. Drug Dev. Ind. Pharm. 15 (6 & 7):1036, 1989.)

Examination of the recent pharmaceutical literature suggests that formulators have now considered most, if not all, routes of administration. Some notable successes have already

occurred. For example, transdermal drug delivery to the general circulation for such drugs as nitroglycerin, estrogen, and nicotine has been both scientifically impressive and commercially successful. The full evaluation of these products has required an interaction between the traditional chemical-based methods of formulation and newer biological methods of pharmacokinetics [13]. Some routes of administration are probably only of use for localized pharmacotherapeutics. For example, it does not seem likely that the vagina will be used routinely as a route to deliver drugs to the general circulation

[14]. A great deal of effort has been expended in studies of the potential of the intranasal route of administration, and considerable ingenuity has been shown in the design of drug delivery systems for this route [15]. However, many pharmaceutical scientists have reservations about how successful the intranasal route will prove to be for administration of drugs to the general circulation on a routine basis. Will the insult, which even the most carefully designed drug delivery is likely to offer to the nasal mucosa, reduce the frequency for which this route can be used? Will absorption be sufficiently reproducible to allow this route to be used for drugs such as insulin? Perhaps the buccal route may have a greater potential.

The emergence of polypeptides and proteins as a class of drugs of increasing therapeutic importance has presented pharmaceutical formulators with an imposing set of challenges. At present, the majority of such drugs are formulated as basically aqueous solutions ready for injection or as freeze-dried powders that require reconstitution before administration. The possible use of the colon as a site of absorption for polypeptides and perhaps even small proteins is now being explored by a number of research groups, and novel methods of processing protein drugs, such as spray drying, are being developed [16]. This area presents many unique challenges to the formulator who must, inevitably, rely extensively on pharmacokinetic techniques to monitor the success of drug delivery systems.

Perhaps the biggest challenge pharmacokinetics has implicitly presented to pharmaceutical formulators has been to produce drug delivery systems, the output of which will respond directly to level of drug in the general circulation of some other relevant bioparameter. Such closed loop systems are extremely attractive, and such devices as microionresponsive electrodes may assist in converting these concepts into practicality. However, one of the biggest blocks that, at present, prevents this type of development is the availability of new pharmaceutical excipients that have generally regarded as safe (GRAS) status and for which Drug Master Files are accessible to those filling INDs or NDAs. The recently formed International Excipients Council is doing a fine job in this area [17], but it would be oversanguine to state that problems in the approvability of new excipients are all likely to be solved within the next few years.

H. Modified Release Drug Delivery Systems

Modified release drug delivery systems have, during the past 30 years or so, attracted a great deal of interest from pharmaceutical companies. A great deal of ingenuity has been applied in the design of many such systems [18], and an examination of the pertinent literature is indeed both instructive and impressive.

As has been pointed out by Grass and Robinson [18], there is some confusion as to the

meaning of terms used in this area. Probably this is due, at least in part, to the role marketing professionals have had in exploiting modified release

products for advertising purposes. As used in this chapter, the author will generally be following the approach proposed by Grass and Robinson for terminology in this field. Immediate release drug delivery systems are those for which no attempt has been made in their design to control the rate at which drug is released from the product. Thus, most immediate (or nonmodified release) drug delivery systems function simply as buckets emptying drug rapidly and indiscriminately into the general circulation. By contrast, a modified release drug delivery system has been specifically designed so that the release of drug is controlled, either temporally or spatially, with the objective of improving safety, efficacy, or patient convenience over that of a conventional nonmodified release drug delivery system.

Enteric-coated tablets or capsules are modified release drug delivery systems that have been used for many years. The rationale behind the use of such systems is that if a drug has reasonable stability at pH values likely to be experienced in the intestine, but has substantial stability problems at pH values likely to be found in the acidic milieu of the stomach, delaying the release of drug delivery system until it has reached the intestine is obviously desirable. Unfortunately, in the past a number of enteric-coated products failed when subjected to pharmacokinetic evaluation. It appears that the enteric coating techniques were insufficiently sensitive to respond adequately to the relatively small pH differential between the stomach and small intestine. Thus, either the drug was not protected in the stomach, or it was not released rapidly enough in the small intestine. As a result of these pharmacokinetic studies, the technology of enteric coating has improved dramatically in recent years, and enteric coating can be much more effective than previously.

However, the most noteworthy developments in modified release products have been the formulation of drug delivery systems designed either to reduce dosing frequency through control of the temporal aspect of drug release or to deliver drug preferentially to target receptors.

Control of temporal aspects of drug release has several obvious advantages, including convenience to the consumer and, more importantly, improvement in the effective therapeutic ratio of the drug by the elimination of peaks and troughs in the plasma concentration/time profiles. If a drug delivery system releases drug at a zero order rate over an extended period, and if the release rate exactly matches clearance, we may expect to obtain blood levels of the drug that remain essentially constant for a considerably period of time. The virtues of this ideal prolonged release product may indeed seem self-evident, and there can be no doubt that when applied effectively and appropriately this approach has much to commend it. However, the development of oral sustained release

products for drugs that pharmacokinetic studies have demonstrated to have long half-lives is not easy to justify, except on marketing grounds. The interesting idea has been advanced that for some drugs a constant level of drug in the bloodstream is not

necessarily ideal, and thus a pulsed pattern may be more effective. Additional pharmacokinetic and pharmacodynamic studies may well be needed in this area before pharmaceutical formulators can devote a major effort to this concept.

Until quite recently temporal control of drug release for more than about a day was generally considered not to be feasible if the oral route of administration was used. However, in theory at least, the use of mucoadhesive drug delivery systems extends our potential to deliver drug over quite long periods of time by the oral route from a single drug delivery system [19] (see Figure 4).

I. Target Organ-Oriented Dosage Forms

With a conventional drug delivery system, the drug released from the formulation is distributed through the body in a manner defined by the pharmacokinetic properties of the drug molecule rather than any specific control factor within the drug delivery system. The majority of orally administered drug products fall into this category. This nonspecificity in the spatial delivery of drug

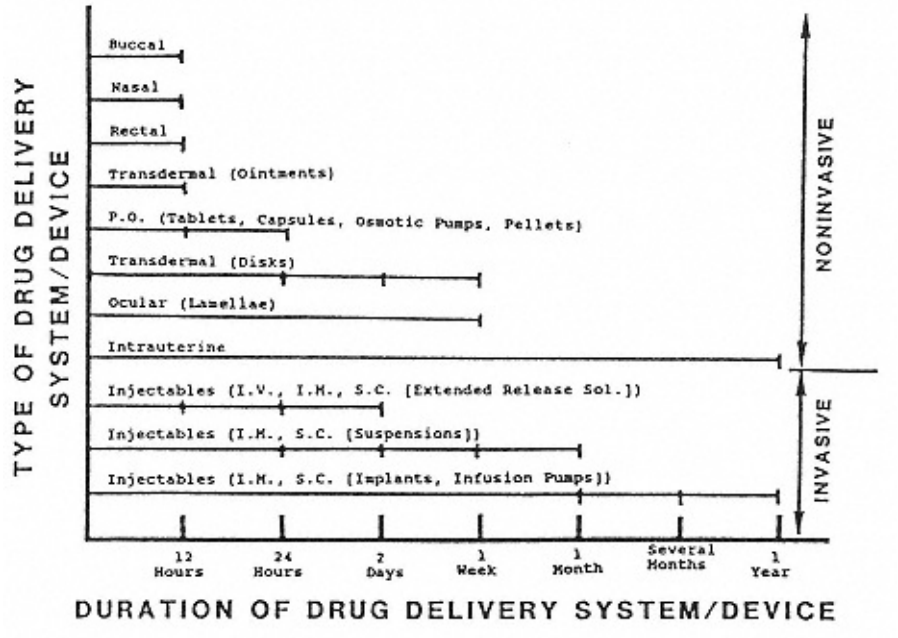

Figure 4
Schematic diagram of types of controlled-release delivery systems/devices, route of administration, and length of duration of action. (From Ritschel. Drug Dev. Ind. Pharm. 15 (6 & 7):1075, 1989.)

within the body has at least two major potential disadvantages. First, even though we may administer quite a substantial dose of drug, the amount that may actually reach the therapeutic receptors may be relatively low, and thus response may be less than optimal. If we were able to get drugs delivered specifically to the therapeutic receptors, we might be able to effect a radical improvement in therapeutic response. Second, by indiscriminate delivery of drug to all parts of the body, we may well exacerbate the occurrence of undesirable side effects, whereas if we were able to limit delivery largely or even exclusively to the target organ, for some drugs at least we may reasonably expect to see a significant reduction in adverse effects. Thus, in total, a target organ-oriented drug delivery system may well result in a substantial improvement in the effective therapeutic ratio of a drug.

The concept of a target organ-oriented drug delivery system is not a 20th century invention. For example, preparations such as cream of castor oil and zinc have been used for many years in the treatment of diaper rash. This product is applied directly to the area of the baby's bottom directly affected by this condition. Generations of teenagers have likewise used preparations based on benzoyl peroxide for direct application to the lesions caused by acne. However, until quite recently target organ-oriented dosage forms relied on the ability of the person applying the product (physician, nurse, or patient) to place the dosage form in close proximity to the affected organ. Thus, we were very largely restricted to the skin or body cavities that could be reached without difficulty. Within the past 20 years, however, pharmaceutical scientists have begun to explore the design of target organ-oriented drug delivery systems in which the dosage itself has some built-in control factor that allows it to direct the drug to the appropriate area of the body [20,21]. One therapeutic class for which target organ-oriented drug delivery systems are of particular potential importance is antineoplastic agents. A large number of new drugs have recently been developed that are remarkably effective in destroying cancer cells. Unfortunately, many of these drugs are also quite toxic to noncancerous cells; thus, in many instances, the use of such drugs is limited because of toxic side effects. In some cases, special measures have to be taken to reverse these side effects. For example, it is normal to give leucovorin rescue therapy after the administration of methotrexate to patients with cancer. One of the earliest attempts in this field involved dispersing drugs in microparticles composed of fats with a melting point of about 38°C. The idea behind this drug delivery system was that since neoplasms are often characterized by a higher metabolic rate, and therefore a higher temperature than normal tissue, the drug would be preferentially released in the vicinity of the cancerous tissue. Other approaches that have been proposed include magnetic direction of microparticles containing drug to the affected organ.

The techniques that probably have the greatest potential, at present, for target organ-oriented drug delivery systems are those in which drug is linked to the fraction crystallizable tail of the monoclonal antibody. The fraction antigen-binding (FAB) head of the monoclonal antibody has specific binding affinity for a surface antigen of the neoplasm. Thus, the drug is preferentially delivered to a site where its activity is desired. The link between the drug and the antibody is of such a type as to break down relatively easily so that free drug is made available for therapeutic action. The development of mixed monoclonal antibodies has added another dimension to target organ oriented drug delivery. The FAB for one-half of the mixed monoclonal antibody is specific for the target organ, and the second FAB is specific for the drug of choice. The mixed monoclonal antibody functions as a beacon, directing the drug specifically to the target organ.

The widespread use of target organ oriented drug delivery systems is still some way in the future, but clearly the potential of this approach is very substantial. The success of such approaches depends on interdisciplinary cooperation among those skilled in formulation, pharmacokinetics, pharmacodynamics, and biochemistry.

III. Conclusion

The nature of pharmaceutical formulation has changed radically within the past 30 years or so. In the early 1960s, formulation was a mix of intuitive art, empiricism, with a topping of science. The proportions of intuitive art and empiricism have declined so that nowadays, in most formulation activities, science predominates. As pointed out in an earlier part of this chapter, developments in pharmacokinetics have played a major role in this trend. Pharmacokinetics provides a benchmark for formulators to evaluate the success of their work and also, in many cases, a road map for them to use when considering novel drug delivery systems or routes of administration. Formulation in the 21st century will continue to rely very heavily on both pharmacokinetics and pharmacodynamics. Indeed, the interaction between these disciplines will require that those entering pharmaceutical formulation as a career have a very strong background in pharmacokinetics. The interaction between pharmacokinetics and formulation is most evident in the development of veterinary pharmaceutical products [21]. Although there are obvious, ethical reasons that may prevent the formulation of human pharmaceutical use attaining the same degree of interdisciplinary cross-fertilization, we can expect much more interaction. As techniques develop for quantification of pharmacodynamic effects, it would seem logical that formulators would make more use of such techniques for the evaluation of test products.

Novel drug delivery systems may well become increasingly important. However, it is my judgment that the orally administered compressed tablet is likely to maintain its position of preeminence as the drug delivery system of choice in North America and Europe well into the 21st century, although there can be little doubt that novel drug delivery systems will attain a considerable degree of success [2022].

Perhaps the most important trend in formulation will be an increasing concern about the ability of drug delivery systems to perform reliably and effectively not just on average to the general population, but on an individual basis to specific patients. Thus, in the evaluation of bioequivalence data a trend is developing for the intrasubject variability to be evaluated as well as average pharmacokinetic data [22]. This trend is likely to continue, and poses additional challenges for pharmaceutical formulators. If this trend does gain momentum, such matters as validation of process are likely to become increasingly sophisticated.

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References

1. D. C. Monkhouse. Drug Dev. Ind. Pharm. 10:1373, 1984.

2. D. C. Monkhouse and A. Maderich. Drug. Dev. Ind. Pharm. 15(13):2115, 1989.

3. S. Reigelman, Dissolution testing in drug development and quality control. The Academy of Pharmaceutical Sciences Task Force Committee, American Pharmaceutical Association, Washington, DC, 1974, p. 31.

4. T. H. Wilson and G. Wiseman. J. Physiol. 123:116, 1954.

5. A. J. Romero, R. R. Rackley, and C. T. Rhodes. Chirality 3:1, 1991.

6. A. J. Romero and C. T. Rhodes, J. Pharm. Belg. 48:2732, 1993.

7. A. J. Romero, L. Savastano, and C. T. Rhodes. Int. J. Pharm. 99:125129, 1993.

8. A. S. Hussain, X. Yu, and R. D. Johnson. Pharm. Res. 8:1248, 1991.

9. P. Veng-Pedersen and N. B. Modi. J. Pharm. Biopharm. 20:397, 1992.

10. M. K. Kottke and C. T. Rhodes. Drug. Dev. Ind. Pharm. 17:1157, 1991.

11. Conference Proceedings, June 1992, International Open Conference on Dissolution, Bioavailability and Bioequivalence. United States Pharmacopoeial Convention, 1992.

12. J. B. Schwartz. In Modern Pharmaceutics, second edition. Edited by G. S. Banker and C. T. Rhodes. Marcel Dekker, New York, 1989.

13. H. Ho and K. W. Chien. Drug Dev. Ind. Pharm. 19:295, 1993.

14. A. A. Deshpande, C. T. Rhodes, and M. Danish. Drug Dev. Ind. Pharm. 18:1225, 1992.

15. D. Duchene and G. Ponchel. Drug Dev. Ind. Pharm. 19:101, 1993.

16. J. Broadhead, S. K. Rouan, and C. T. Rhodes. Drug Dev. Ind. Pharm. 18:1169, 1992.

17. L. Blecher. Pharm. Tech. June:55, 1991.

18. G. M. Grass IV, and J. R. Robinson. In Modern Pharmaceutics, second edition. Edited by G. S. Banker and C. T. Rhodes. Marcel Dekker, New York, 1989.

19. R. Jimenez-Castellanos, H. Zia, and C. T. Rhodes. Drug Dev. Ind. Pharm. 19:143, 1993.

20. A. Kydonieus (Ed). Treatise on controlled drug delivery. Marcel Dekker, New York, 1991.

21. J. Blodinger (Ed). Formulation of Veterinary Drugs. Marcel Dekker, New York, 1983.

22. FDA Generic Drugs Advisory Committee, public hearing, 1012 February, 1993.

23. M. R. Jimenez-Castellanos, H. Zia, and C. T. Rhodes, Int. J. Pharmaceutics 105: 6570, 1994.

4
Peptide and Protein Drug Delivery

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I. Introduction

Peptide and protein drug delivery has been dominating pharmaceutical and biomedical research for more than a decade and appears to continue doing so in

the forseeable future. These are exciting times in drug delivery because of the rapid changes in the fundamental approaches in this endeavor that are about to take root. Thus, the oral route will no longer be the exclusive route of administration; other mucosal routes such as the nasal, pulmonary, buccal, and transdermal routes may, in fact, hold the key to the ultimate success of peptide and protein drugs in therapeutics. Passive diffusion mechanism has likewise seen its limitations as a guide in the design of peptide drugs that are readily absorbed. Nonpassive transport mechanisms, including carrier-mediated transport and transcytosis, must also be considered. Other new drug delivery concepts that are emerging include pulsatile drug release as an alternative to zero order release, use of penetration enhancers to facilitate peptide and protein drug absorption, and development of targeted drug release systems for oral use. The focus of this chapter will be on the pharmacokinetic barriers to peptide and protein drug delivery and means to overcome them. These barriers include penetration, preabsorptive metabolic clearance, and postabsorptive hepatic and renal clearance. The delivery of genes in both viral and nonviral vectors as a means to circumvent many of the protein delivery problems [1] will not be discussed.

II. Penetration Barriers to Peptide and Protein Drug Delivery

The mucosae lining all the routes being considered for peptide and protein drug delivery, except the oral route, are not meant to be absorptive in nature. It is, therefore, not surprising that considerable resistance to transport has been experienced by most peptide and protein drugs. The bioavailability of an octapeptide dynorphin analog, E-2078 (m.w. 1035), following rectal administration in the rat was only 22%, compared with 68% following subcutaneous and intramuscular administration [2]. This was, however, better than the 1% for leuprolide [3] and the 0.8% for calcitonin [4]. Lowering the pH to 5 and increasing the viscosity to 1,000 cP brought about a three- and twofold increase in the bioavailability, respectively. On the other hand, there was no change in the bioavailability upon lowering the osmolality to 0 mOsm/kg or raising it to 2000 mOsm/kg. Unlike other mucosal routes, the uterus of the rat is surprisingly permeable to insulin and calcitonin [5]. Golomb et al. [5] found that the extent and duration of the respective pharmacodynamic response induced by the intrauterine delivery of these two hormones were equivalent to those obtained after subcutaneous injection. The reason for the excellent bioavailability was not determined, however.

Resistance to peptide and protein drug transport can be due to lipophilicity of the barrier, its tight junctional resistance, or its low capacity for carrier-mediated or endocytotic transport. Before addressing the individual contribution of each of these barriers, it would be useful to comment on the possible role

played by the mucus layer in drug transport. This layer comprises a heterogeneous group of proteins, chief among which are mucus glycoproteins. Its thickness ranges from 80 mm in rat duodenum [6] to 180 mm in mouse colon [7]. In addition to its protective role, it also serves to maintain an inwardly directed pH gradient [8], which is crucial to H+ - dipeptide cotransport. Invoking the concept of phase separation of polymer solutions, Edwards [9] proposed that protein molecules would be excluded from the mucus unless they possess a surface structure that favors interaction with the mucus glycoproteins. In this way, cells are protected against the entry of bacteria, toxins that they may produce, viruses, and protein drugs.

Although of limited importance in the diffusion of various nutrients and drugs [10], the definitive role of mucus as a penetration barrier to peptide and protein drugs is unclear. This phenomenon can now be studied in detail using the mucus layer producing human intestinal goblet cell line HT29-H [11]. This cell line forms confluent monolayers that contain mainly mature goblet cells. Compared with Caco-2 cell monolayers, HT29-H monolayers are four times lower in transepithelial resistance (100 W.cm2 vs. 410 W.cm2) and 53 times more permeable to mannitol, a paracellular marker [11]. Nevertheless, testosterone, a lipophilic drug, is only 43% more permeable than mannitol across HT-29H monolayers when compared with a 38 times difference in the permeability in Caco-2 cells, suggesting that mucus may play a rate-limiting role. Such a possibility is substantiated by the twofold increase in testosterone transport after removal of the mucus layer. It is encouraging that the complexation of insulin with dodecylmaltoside in a 1:4 weight ratio improved the compatibility of insulin with mucus, while at the same time stabilizing it against degradation by pepsin and chymotrypsin and against self-aggregated precipitation on surfaces [12]. The net result was a threefold increase in the systemic bioavailability of insulin when administered intraduodenally in the rat.

A. Types of Barriers.

1. Tight Junctional Resistance

The tight junctions or zonula occludens are a dynamic structure that regulate the paracellular permeability to solutes [13]. Because of the 0.1% of the total surface area of the absorptive epithelium it contributes to absorption [14], the paracellular pathway of transport has thus far been ignored as a drug transport pathway. Under physiological conditions, the molecular weight cutoff in the ileum and colon appears to be in the range of 400500 [15]. The nasal epithelium is as leaky as the small intestinal epithelia, and is 10 times leakier than the rectal mucosa and 5 times leakier than the vaginal mucosa [16]. By contrast, the buccal epithelium is at least 25 times tighter on the basis of transepithelial resistance [17]. Second only to the skin in terms of resistance to polar drug

transport [18], the molecular weight cut-off of the buccal mucosa is surprisingly high. Using confocal laser scanning microscopy, Hoogstraate et al. [19] detected the passage of 20 kD FITC-dextran into the porcine buccal epithelium. The same technique has revealed the exclusive passage of FITC-octreotide into the intercellular space of cultured monolayers of cerebrovascular endothelial cells [20].

An inverse relationship has been reported by McMartin et al. [21] and Kubo et al. [22] for drug transport across the nasal mucosa of the rat and rabbit, respectively. Using an Using chamber arrangement, Kubo et al. [22] reported that the permeability of FITC-dextrans of varying molecular weights (m.w. 4,400, 9,400, 35,600, and 71,200) was 230 times lower than that of hydrophilic sodium cromoglycate but was 20200 times lower than that of lipophilic propranolol. Yamamoto et al. [23] reported that improvement in the nasal absorption of macromolecules at the upper limit of the molecular weight range was marginal, however aggressive was the enhancer. As expected, molecular size is not the sole determinant of paracellular permeability. Molecular shape should also be considered. Thus, polyethylene glycol (PEG) with a m.w. of 194 was 6- to 28-fold higher in permeability than mannitol of comparable molecular weight (m.w. 182) [15]. The investigators speculated that reptation motion by PEG through the narrow tight junctions may be the explanation. Finally, in addition to size and shape, the penetrant's charge also plays a role in paracellular transport. Since the tight junction carries fixed negative charges [24], cationic solutes are preferred.

An attractive feature of paracellular transport is that the penetrant may be spared from the deleterious influence of intracellular proteases. However, the structural features of peptides that favor paracellular transport are as yet unknown. Work by Yen and Lee [25] suggests that 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (Pz-peptide) may be a peptide that deliberately seeks out the paracellular pathway for transport [25]. This peptide is designed to be susceptible to the action of collagenase-like enzymes at the Leu-Gly bond, while resisting the action of aminopeptidase and carboxypeptidase by virtue of the N-terminus Pz group and the C-terminus D-arginine group, respectively [26]. In spite of its susceptibility to intracellular proteolysis, Pz-peptide is able to penetrate various intestinal segments of the albino rabbit essentially intact without the use of protease inhibitors and penetration enhancers [25]. An equally impressive feature of Pz-peptide is that in spite of its hydrophilicity it penetrates all intestinal segments of the albino rabbit as well as, or better than, atenolol, timolol, and propranolol of higher lipophilicity.

A possible explanation for the ability of Pz-peptide to escape the surveillance of intracellular collagenases is that it opts for the paracellular pathway of transport. Although

the tight junctions in the upper intestinal segments (1015 Å) may be leaky enough to permit the passage of Pz-peptide, such may not be the

case in the lower intestinal segments (46 Å) [27]. The higher apparent permeability coefficient for Pz-peptide in the lower intestinal segments, despite a higher susceptibility to intracellular proteolysis, therefore, indicates that Pz-peptide must be able to enhance tight junctional permeability, thereby facilitating its own penetration. An indication of enhancement in paracellular permeability is a fall in the transepithelial resistance (TEER). This has been shown to be the case in the nasal mucosa [22,28]. It must be pointed out that, in using the TEER as a measure of paracellular permeability in tighter epithelia such as the colon, the contribution of transcellular ion flow to TEER cannot be ignored [15].

The mechanism by which Pz-peptide increases junctional permeability is as yet unknown. There are at least two possibilities. One possibility is that Pz-peptide promotes net ion absorption thereby triggering a solvent drag effect, as is the case in enhancement by glucose and amino acids of the paracellular permeability of the hamster small intestine to PEG 4000, inulin, and a hemeconjugated peptide tracer (m.w. 1900) [29,30]. However, this solvent drag effect does not appear to be very prominent in the rabbit small intestine, as indicated by the lack of increase in mannitol and atenolol transport in the presence of Pz-peptide. Therefore, the more pronounced increase in junctional permeability by Pz-peptide in the large than the small intestine may conceivably be due to the tighter nature of the large intestine [31] and hence its greater sensitivity to the solvent drag effect induced by Pz-peptide. The latter possibility is consistent with the water reabsorption function of the large intestine [32]. Recent findings have revealed that Pz-peptide probably stimulates, either directly or indirectly, the Na^+ channel on the apical membrane, hence transepithelial Na^+ flux, water movement, and tight junctional permeability (Yen and Lee, unpublished data).

As another possibility, Pz-peptide may increase junctional permeability by entering the cell so as to mobilize the release of Ca^{2+} from its intracellular stores, an event preceding contraction of the actomyosin filaments associated with opening of tight junctions [33]. Recent evidence suggests that this may indeed be the case (Yen and Lee, unpublished data). Llopis et al. [34] demonstrated that an increase in intracellular Ca^{2+} led to microvilli contraction and an increase in the transport of horseradish peroxidase across the tight junctions in the perfused rat liver.

2. Resistance to Transcellular Transport

a. Simple Diffusion. On the basis of potential surface area available for drug transport, it is reasonable to expect that the majority of drug molecules will opt for the transcellular route for transport. For the majority of nonpeptide drugs, lipophilicity is the principal determining physicochemical drug property. Such a paradigm does not appear to serve peptide drugs very well. Based on transport studies on Caco-2 cells using a series of small

peptides of varying lipophilicity, chain length, and number of polar functionalities, Conradi et al.

[35] proposed that a peptide must possess a delicate balance of affinity for the aqueous-membrane interface (favorable log $PC_{n\text{-octanol/buffer}}$ partition coefficient) and a reasonably low desolvation energy (favorable log $PC_{heptane\text{-}ethylene\ glycol}$) in order for it to cross an epithelial cell membrane efficiently, including the blood-brain barrier [36]. Thus, a peptide with a large n-octanol/buffer PC and large desolvation energy due to a significant number of polar groups should adsorb and remain at the membrane interface. Only within a narrow range of lipophilicity would increased drug lipophilicity lead to enhanced drug penetration. This is the case for the peptidomimetic angiotensin converting enzyme inhibitors zofenoprilat, enalaprilat, ramiprilat, and fosinoprilat [37].

There is circumstantial evidence that a potentially formidable obstacle to lipophilic peptide transport resides in the p-glycoprotein gp170, a 170-kD transmembrane ATP-dependent drug efflux pump [38] first identified in multiple drug-resistant tumor cells [39] and subsequently in the mucosa of the rat small intestine [40]. It has exquisite affinity for such lipophilic substrates as daunomycin [40], progesterone [41], and vinblastine [42]. More recently, gp-170 has been implicated in contributing to the poor penetration of cyclosporin A [41] and the lipophilic peptide AcPhe $(NMePhe)_2NH_2$ [43] in Caco-2 cells. For both peptides, transport was lower from the apical to basolateral direction than in the opposite direction and was concentration dependent in the apical to basolateral direction. The incorporation of 100 μM of chlorpromazine and progesterone, gp170 pump inhibitors, increased the apical to basolateral transport rate of cyclosporin A (0.5 μM) approximately threefold while reducing that in the opposite direction to 25% of the control [41]. The incorporation of 300 μM verapamil, another gp170 pump inhibitor, likewise improved the apical to basolateral transport of AcPhe $(NMePhe)_2NH_2$ [43] two times. These interesting findings raise the equally interesting possibility that the gp170 drug efflux pump may also exist in other mucosal routes, thereby limiting lipophilic peptide absorption. Therefore, a strategy to improve mucosal peptide drug penetration will be to design pharmacologically inert inhibitors of the gp170 drug efflux pump or to design peptides that are poor substrates for this pump. Such an undertaking would require parallel concerted efforts to explore the existence of other, and possibly more potent, drug efflux pumps and to delineate their molecular and biochemical characteristics.

b. Carrier-Mediated Transport. Peptide carriers have usually been studied relative to their role in the intestinal absorption of di- and tripeptides formed from luminal digestion of dietary proteins. There is as yet no definitive evidence for their existence in any absorptive mucosa being considered for peptide drug administration, except the alveoli of the lung [44]. Driven by a transmembrane H^+ rather than Na^+ gradient, this peptide carrier is involved in the intestinal transport of several cephalosporins [45,46], angiotensin-

converting enzyme inhibitors [47,48], and perhaps others.

Based on their structure, di- and tripeptides with a large n-octanol PC are preferred substrates for transport by this transporter [49]. The other structural requirements are both a free amino and carboxyl terminus, the amino group and peptide bond nitrogen located in the a-position, a *trans*- rather than a *cis*-peptide bond, and L-a-amino acid isomers in both COOH and NH_2 termini, although D-isomers of hydrophobic amino acids are acceptable in the NH_2 terminus. These requirements are not absolute. Indeed, on the basis of competitive inhibition of the transport of a series of dipeptide analogs without the N-terminal a-group in the perfused rat intestinal segment by Gly-Gly, Gly-Phe, Pro-Phe, Gly-Pro, and cephradine, a free a-amino acid group has been found not to be absolutely essential for mucosal-cell peptide carrier-mediated transport [50]. Such a characteristic of the dipeptide carrier appears to be beneficial from the peptide drug design point of view.

Progress in the design of peptides with affinity for the peptide carrier is expected to be slow unless the putative peptide carrier is purified and its gene cloned. Since 1987, two groups have started the isolation and purification of the peptide carrier protein, but progress has been painfully slow. Iseki et al. [51] obtained three components (fractions a, b, and c) from the 105,000 × g supernatant of the rat intestinal mucosal homogenate using tandem ion-exchange and gel-filtration chromatography. All three fractions, and particularly fraction b with a m.w. of 15,000 [52], were found to bind several b-lactam antibiotics, including ampicillin, amoxicillin, cyclacillin, cephalexin, cephradine, and cefadroxil. Whether binding to fraction b would lead to uptake and transport was not determined. The same question can be posed to the much higher molecular weight protein (m.w. 127,000), purported to be the peptide carrier, which was discovered in the rabbit intestine by Kramer et al. [53]. This protein was identified in the brush border membrane vesicles using [3H]benzylpenicillin photoaffinity labeling and sodium dodecyl sulfate (SDS) gel electrophoresis. Gly-Pro, b-AlaHis, and Pro-Gly were found to reduce the labeling of this protein by [3H]benzylpenicillin.

To circumvent the difficulty associated with purification of the peptide carrier, Miyamoto et al. [54] devised an alternate strategy involving the identification and purification of the mRNA encoding the intestinal peptide carrier. This strategy involves the use of *Xenopus laevis* oocytes to express the peptide carrier in a functionally competent form upon microinjection of exogenous poly(a)+ mRNA isolated from the rabbit intestinal mucosal cells into the oocytes. The functional competence of the expressed carrier was probed using Gly-Sar as a substrate. Compared with water-injected oocytes, poly(A)+ mRNA-injected oocytes exhibited a threefold higher dipeptide uptake activity. Expression of the carrier was not evident until day 2, increasing with time and reaching a plateau at day 4. The expressed carrier retained its dependence on a transmembrane H+ gradient for maximum activity as well as its substrate specificity, having affinity

for the dipeptides Gly-Sar and Gly-Pro and no or little affinity for the constituent free amino acids.

This pioneering work of Miyamoto et al. [54] was subsequently corroborated by Fel et al. [55] in the rabbit small intestine. A 707-amino acid protein with 12 transmembrane domains and an unusually large extracellular hydrophilic loop where N-linked glycosylation occurs, this transporter is abundantly expressed in the small intestine (duodenum = jejunum > ileum), at a lower level in the colon, kidney, and liver, and only weakly in the brain. It has broad substrate specificity, transporting di-and tripeptides as well as cephalosporin antibiotics but not amino acids and tetrapeptides. Peptides containing bulky aliphatic side chains are especially preferred. Uptake mediated by the transporter is electrogenic, due to the cotransport of H^+. But it is completely independent of extracellular Na^+ and Cl^-.

The cloning and expression of the intestinal peptide-proton cotransporter just described are very exciting developments in peptide drug delivery. An aspect of peptide transport that deserves equal attention is the mechanism by which the peptide molecules already transported across the brush-border membrane are eventually exported across the basolateral membrane. Work by Dyer et al. [56], Inui et al. [57], and Thwaites et al. [58] suggests that a carrier distinct from its counterpart in the apical membrane may be involved in the transfer of dipeptides across the basolateral membrane in rabbit intestinal cells and Caco-2 cells. In Caco-2 cells, this is a temperature-dependent process driven by an inwardly directed pH gradient that is sensitive to perturbation by sulfhydryl-active agents, such as PCMBS [57,58]. In rabbit enterocyte basolateral membrane vesicles [56], the uptake of Gly-Pro is also stimulated by the presence of an inwardly directed H^+ gradient ($pH_i = 7.5$, $pH_o = 5.5$). This stimulation is abolished by the proton ionphores carbonyl cyanide p-trichloromethoxyphenylhydrazone (CCCP) and tetrachlorotrifluoromethylbenzimidazole (TTFB) at 1 mM. Gly-Pro transport (10 mM) is also inhibited by 1 mM each of $HgCl_2$ and thimerosal. At 1 mM, Gly-Pro transport is inhibited to varying extents by 10 mM dipeptides, including Gly-His, cephradine, carnosine, Gly-Gly, and Gly-Phe. Gly-Glu is, however, not inhibitory. Further work is necessary to characterize fully the basolaterally located peptide carrier, including its relationship with the apically located peptide carrier.

c. Endocytosis. Endocytosis is a complex multistage process comprising the capture of molecules present in the fluid phase or bound to the cell surface to form vesicles, which are then routed and sorted to various destinations within the cell for further processing, including proteolysis in the lysosomes [59]. b-Lactoglobulin (BLG, m.w. 35,000) has been observed to be absorbed into epithelial cells of cultured human duodenal explants by

endocytosis [60]. The number of BLG-immunopositive epithelial cells appeared to increase with time, as did their immunoreactivity. At 30 min immunopositive cells were more often

present toward the lower thirds of the villi than at the villus tips and occasionally below the crypt villus junction. This pattern is consistent with the higher endocytotic activity in the relatively immature crypt than villus cells [61].

Simionski et al. [62] reported that nerve growth factor, when injected into the ileal loop of the suckling rat, was transported across the epithelium by both receptor-mediated and nonspecific endocytosis to reach the systemic circulation intact. Some degradation did occur in the lysosomes, however. It is possible that the cationic nature of the protein (pI 9.3) might have facilitated its interaction with the absorptive surface and subsequent transcytosis.

A scavenger receptor-mediated mechanism that recognizes polyanions above a certain molecular weight threshold, similar to the one that exists in the liver [63], appears to exist in cultured brain microvessel endothelial cells [64]. However, it is not certain if a similar mechanism exists in epithelial cells that can be exploited to facilitate protein absorption.

B. Means to Overcome Penetration Barriers

1. Chemical Modification

The literature is replete with examples of various approaches by which peptides are modified to improve penetration efficiency as well as metabolic stability. A recent development of note is that based on the prodrug approach [65]. This approach involves the bioreversible derivatization of a parent peptide that possesses the functional groups amenable to modification. Kahn et al. [66] tested this concept on desmopressin, a synthetic analog of the antidiuretic hormone vasopressin used in the treatment of central diabetes insipidus and nocturnal enuresis and as a hemostatic agent. Various aliphatic carboxylic acid esters and a carbonate ester of the tyrosine phenolic group in desmopressin were synthesized. All esters were quantitatively converted to desmopressin in human plasma and rabbit liver homogenates. Being more lipophilic than the parent peptide, all esters penetrated across the Caco-2 cell monolayers well. However, there was no obvious correlation between the permeability of the ester prodrugs and their lipophilicity. In contrast to this approach, which relies on reactive functional groups on amino acids in the backbone, Muranishi et al. [67] tested an alternative approach based on the attachment of fatty acid moieties to the amino termini of peptides. Not surprising is that some loss of pharmacological activities has been observed. However, this may be offset by improved proteolytic stability and improved penetration across the intestinal mucosa. Thus far, such an approach has been applied to thyrotropin-releasing hormone, tetragastrin, and insulin.

2. Use of Penetration Enhancers

By far the most widely investigated means to enhance peptide and protein drug absorption is that based on penetration enhancers. These are low molecular

weight compounds that enhance drug penetration by lowering the diffusional resistance across either the paracellular or transcellular pathway, or both [68,69]. Examples include bile salts [23,70,71], chelators [23,72], surfactants [7375], salicylates [76,77], enamines [78,79], glycosides [80], fusidate derivatives [8184], cyclodextrin derivatives [85,86], acylcarnitines [87], fatty acids [88,89], and phospholipids [90,91]. The mechanism of action is diverse, ranging from Ca^{2+} sequestration in the case of fatty acids [88], to dissociation of oligomers of proteins prone to self-association in the case of bile salts [72,92,93], and to induction of solvent drag effect in the case of D-glucose [94,95]. The mechanism based on stimulation of endocytosis, which is promising in the facilitation of gene transfer [96] and macromolecular drug transport across the blood-brain barrier [97] and the intestinal epithelium [98], deserves more attention in the future. It is intriguing that the infection of fibroblasts with the Friend retrovirus primed the cells for increased activity in internalizing liposomes [99].

A particularly interesting absorption enhancer is the sodium salt of decanoic acid (Na caprate, C10), which is used in Japan to enhance the absorption of drugs in humans [100]. C10 is relatively well studied in comparison to many other absorption enhancers. Sawada et al. [101] determined that, as was the case for a 1:3 mixture of soybean phosphatidylcholine and medium chain monoacylglycerol [90], this enhancer acted mainly on the paracellular pathway in the rat colon. This enhancer is effective only above 4 mM. The existence of such a threshold is attributed to its complexation with extracellular Ca^{2+} [102]. In Caco-2 cells, the effect of C10 on transepithelial resistance is immediate. This effect is accompanied by structural deformations of the tight junctions in the form of dilatations. Anderberg et al. [102] speculated that these structural dilatations in the tight junctions could be the consequence of mobilization of intracellular Ca^{2+} and contraction of the perijunctional actin ring.

Hastewell et al. [103] found that mixed micelles comprised of 40 mM each of monoolein and Na taurocholate enhanced the colonic absorption of human calcitonin (hCT; m.w. 3,418) in the rat 12 times. Peak hCT concentration was reached within 5 min. However, the bioavailability was not determined. Lower concentrations of mixed micelles were found not to be effective. At 40 mM, absorption of the transport markers, PEG4000 (m.w. 4000) and HRP (m.w. 40,000), was also enhanced by six and nine times, respectively. This potential lack of specificity for the lipidic class of absorption enhancer raises an obvious toxicity concern that needs to be addressed. Na taurocholate is probably a transcellular enhancer [72], whereas monoolein is probably a paracellular enhancer [104]. The mechanism by which monoolein enhances paracellular permeability is probably mediated by its inhibitory action on diacylglycerol kinase [104], thereby elevating the intracellular concentration of diacylglycerol that regulates tight junctional assembly [105].

The extent of penetration enhancement afforded by various enhancers varies. Sodium tauro-24,25-dihydrofusidate (STDHF), 0.5%, improved the nasal absorption of salmon calcitonin administered in a 100 ml spray in human volunteers from 1.6% to 3.97.9% [106]. Polyoxyethylene(24)-cholesterol-ether, 1%, enhanced the oral absorption of octreotide in the rat 23 times from its control value of 0.3% [107]. A eightfold increase was observed in human volunteers. A 1:3 mixture of soybean phosphatidylcholine and medium-chain monoacylglycerol at a total concentration of 35 mM increased the relative bioavailability of low molecular weight heparin (m.w. 5000) following rectal administration in the rabbit from <1% to 21 ± 12% [90]. The extent of enhancement could be adjusted by varying the relative proportion of the two lipidic components. Lohikangas et al. [108] found that monoacylglycerol enhanced whereas phosphatidylcholine inhibited the epithelial permeability enhancement effect of the overall mixture.

There are four concerns associated with the use of penetration enhancers: irritancy, nonspecificity in action, adverse effect on ciliary movement, and absorption of the enhancer into the bloodstream [88]. Nonspecificity in action usually manifests in leaching of membrane proteins [109] and enhancement efficacy relatively independent of the molecular weight of the penetrants [110]. The above concerns are usually either downplayed or ignored at the early stages of development of any penetration enhancer. One of the latest examples is lysophosphatidylcholine [111]. Capable of modifying G protein-dependent signaling in porcine endothelial cells [112], this prospective penetration enhancer has recently been found to cause cellular membrane damage in the erythrocyte hemolysis model [113]. Such damage can, however, be minimized with a concomitant loss in enhancement potency by complexation with hydroxypropyl-b-cyclodextrin in a molar ratio of 1:12 [91]. Another example is sodium taurodihydrofusidate [82]. In spite of its minimal hemolytic effect [82], it was subsequently reported to elicit unacceptable stinging sensations and lacrimation when given as a nasal spray to healthy human subjects [114].

In addition to the above four safety concerns, the selection of penetration enhancers should also consider physicochemical compatibility between the enhancers and the penetrants. Indeed, Lee et al. [115] demonstrated that it was the specific interaction between the basic side chains of 24,25-dihydrofusidic acid and insulin and human growth hormone that led to a diminished enhancement effect afforded by the parent enhancer molecule.

3. Use of Iontophoresis

Iontophoresis usually refers to the transfer of ionic solutes through biological membranes under the influence of an electric field. This technology is being considered as an

alternative to penetration enhancers for the facilitation of transdermal drug delivery. The attractiveness of the transdermal route is the vast

surface area that can be utilized in chronic drug therapy, a definite advantage in instances in which there are frank skin reactions [116,117].

Among the therapeutic peptides and proteins that have been investigated for iontophoretic delivery include thyrotropin-releasing hormone [118], luteinizing hormone-releasing hormone [119,120], insulin [121,122], and arginine vasopressin [123]. The formulation and electrode design factors that optimize iontophoretic drug delivery have been identified [124]. The pathway of drug entry under iontophoresis was once thought to be occurring exclusively at the appendages, such as sweat ducts and hair follicles [125]. Such a view was recently challenged by Monteiro-Riviere [126], who observed drug passage across the intact stratum corneum via the intercellular route under iontophoresis. During iontophoresis under neutral pH conditions, there is a net convective flow of volume (electroosmosis) from anode to cathode leading to enhanced transport of dissolved polar (but uncharged) solutes in the same direction [127,128]. Electroosmotic flow increases with applied current density [129].

A recent study on human epidermal membrane by Inada et al. [130] has revealed that the application of a DC voltage (2504000 mV) reduced the electrical resistance, consistent with pore formation. The rate of decrease in resistance was strongly dependent on the applied voltage. The reversible recovery times were dependent upon both the magnitude and the duration of the applied field, but were frequently several orders of magnitude greater than those for attaining significant resistance reduction. Reversible recovery times were much longer when lower voltages were applied for longer times to attain the same decrease in electrical resistance than for higher voltages at short times. Using the isolated perfused porcine skin flap model, Heit et al. [131] demonstrated that prior exposure of the skin to an iontophoretic current enhanced the flux of subsequently applied luteinizing hormone-releasing hormone (LHRH). Moreover, subsequent iontophoretic episodes resulted in widely divergent fluxes. In addition to the direct effect of the current on the skin architecture, the peptide itself may also modify the skin, particularly if it is lipophilic. Delgado-Charro and Guy [129] made the intriguing observation that nafarelin, an LHRH analog, bound to the skin following iontophoresis sufficiently tightly to alter its permselectivity, whereas LHRH itself did not. This finding explains the loss in transport efficiency with applied peptide concentration in the anode donor chamber.

4. Maximizing the Surface Area for Absorption

The flux across a given mucosa is determined not only by the permeability but also by the surface area. Improved contact with the absorptive mucosae is perhaps one of the several explanations for the approximately twofold increase in the oral bioavailability of cyclosporin in human volunteers when administered as a microemulsion [132]. The

lungs, with their vast surface area of 170 m_2, are an attractive venue for peptide and protein drug delivery. Additional attractive

features are a thin epithelial barrier (1 mm), extensive vascularization (blood flow being about the same as the cardiac output) [133], possibly lower proteolytic activity [134137], and the capacity of alveolar epithelial cells, the principal absorptive cells, to transport peptides and proteins by all known transport mechanisms. Thus, Gly-L-Phe is transported by a carrier-mediated mechanism, attaining a Papp of 1.4×10^{-7} cm/s at 1 mM and 0.7×10^{-7} cm/s at 10 mM [44]. Gly-D-Phe, on the other hand, permeates the alveolar epithelial monolayer by a paracellular simple diffusion mechanism. A carrier-mediated process with a Km of 331 ± 163 mg and Vmax of 90.1 ± 10.9 mg per 30 min was postulated to be responsible for the absorption of a 7 kD oligomer of 2-aminoethylcarbonyl-6-aminofluorescein-labeled poly-a,b-[N(2-hydroxyethyl)-DL-aspartamide] (FPHEA) from the isolated rat lung [138]. However, in light of the molecular size of the penetrant, endocytosis was a more probable mechanism.

Various peptide and protein drugs have been evaluated relative to pulmonary delivery, and their absolute bioavailability averages about 20%. Insulin, when aerosolized to the rat, was rapidly absorbed into the bloodstream, reaching a peak within 12 min and achieving a bioavailability of 57% [139]. The most encouraging result to date is the approximately 62% bioavailability of a recombinant methionyl human granulocyte colony-stimulating factor (rhGCSF) dose instilled intratracheally in golden syrian hamsters. Absorption was rapid, some 20% of the absorbable dose being present in the serum 6 min postinstillation with the peak occurring within 12 hr. The absorption mechanism was not elucidated, but it probably involved transcytosis. In comparison, the bioavailability of rhGCSF in the rabbit following nasal administration in a pH 4 formulation was only about 10% [140]. Machida et al. [141] observed a threefold increase in nasal systemic bioavailability in the presence of 1% polyoxyethylene-9-lauryl ether, but no change in bioavailability in the presence of 1% Na glycocholate.

Various means have been explored to maximize peptide and protein drug absorption from the lungs. Altering the formulation pH represents one such strategy. Okamura et al. [142] reported that the bioavailability of insulin was threefold greater at pH 3 than at pH 7 following intratracheal administration in the rat. Smith et al. [143], on the other hand, detected no change in bioavailability of an RGD tetrapeptide upon lowering the pH from 5.5 to 2.5, although the peak time was reduced and the peak concentration was increased.

Proteins such as insulin and growth hormone require tight control over the administered dose; this underscores the need for a reproducible delivery system. A striking 10-fold difference in insulin bioavailability following intratracheal instillation (5.6%) and aerosolization (57.2%) to the rabbit was observed [139]. The more favorable results with aerosolization can be explained by the larger proportion of the applied dose reaching the

periphery of the lung. It was surprising that aerosolization afforded the same systemic bioavailability of di-

tirelix (m.w. 1,540) in the mongrel dog [144] and the sheep [145] when compared with intratracheal instillation. Both methods of administration led to a bioavailability of 29 ± 10% in the dog and 9.8 ± 3.9% in the sheep with an unusually slow absorption rate (t^{max} = 6.5 ± 3.6 h in the dog and t^{max} = 2 h in the sheep).

Based on a mathematical model for inhaled aerosolized drugs that has been validated against adult subject inhalation tests, Martonen and Katz [146] showed that by altering breathing profiles, deposition values can be affected regarding quantity delivered and spatial location. Increased tidal volumes and breathholding times increase deposition in the pulmonary region, while increased inspiratory flow rates increase deposition in the tracheobronchial region [146]. As the tidal volume varied from 500 to 2000 ml, lung deposition increased monotonically for all particle sizes, and by as much as 100% over a wide size interval (i.e., 0.033 mm). Increased lung deposition is a result of deeper particle penetration into the lung at the increased tidal volumes and the increased particle residence times in peripheral airways associated with the respective breathing profiles. This is an area that merits more work as interest in dry powder formulations grows. An important factor to consider here is the rate of dissolution. This factor has been speculated to explain the 50% lower bioavailability for thyrotropin-stimulating hormone, follicle-stimulating hormone, and human chorionic gonadotropin when given as a powder compared with a solution [147]. Where sustained delivery is desirable, microspheres may be considered. Masinde and Hickey [148] have fabricated microspheres from poly(L-lactic acid) with a median projected area diameter of 4.2 mm for nebulization to the lower airways.

The feasibility of using penetration enhancers to overcome the poor intrinsic permeability of the alveolar epithelium [149] has been investigated. Komada [147] observed a 5- to 30-fold increase in the systemic bioavailability of various peptides following intratracheal instillation in the presence of 50 mM Na glycocholate in rats. The largest increase was seen in human chorionic gonadotropin with the lowest intrinsic bioavailability (0.2%) and the smallest increase was seen in insulin with the highest intrinsic bioavailability (13.1%). Lowering the formulation pH to 3 also resulted in an increase in systemic bioavailability, but to a lesser extent. In a separate study, the incorporation of 20 mM Na glycocholate was found to afford a ninefold increase in bioavailability over the control of 9.1% without noticeably disrupting the integrity of the epithelial cells [150]. This observation prompted the postulation of the mechanisms of bile salt-mediated pulmonary insulin absorption as being a combination of factors such as dissociation of insulin oligomers, dilatation of pulmonary epithelial tight junctions, surface tension reduction, aminopeptidase inhibition, and ciliostatic effect. Nevertheless, from the safety point of view, the effect of the bile salt on alveolar surfactant film stability needs to be ascertained.

Oleic acid, a common ingredient in aerosol formulations, can induce both trans- and paracellular permeability changes at the alveolar epithelium without detectable cellular damage, provided that the concentration is less than 50 mM [151]. Increases in paracellular permeability in cultured rat alveolar epithelial monolayers have been attributed to an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) due to Ca^{2+} influx. Esterified oleic acid (oleic methyl ester) and stearic acid (a saturated fatty acid with equal chain length) had much reduced effect on both $[Ca^{2+}]_i$ and permeability alterations. Degree of unsaturation was apparently unimportant since linolenic acid (18:3), lineolic acid (18:2), and oleic acid (18:1) had similar and comparable effects on the two parameters. Given the uncertainty of long-term safety associated with chronic use of penetration enhancers, a more fruitful approach to enhancing systemic drug bioavailability following pulmonary administration seems to be the engineering of devices that exceed the 20% delivery efficiency plateau of currently available metered-dose inhalers [152].

A potential complication associated with the pulmonary route is loss of stability of the protein during aerosolization, including irreversible conformational changes in the protein structure and oxidative changes through the hugh air-water interface that is repeatedly generated. A case in point is interferon-alpha [153]. This is, however, not a concern with recombinant human deoxyribonuclease I (rhDNase) [154] that was recently approved for improving the clearance of purulent sputum from the airways of patients with cystic fibrosis [155]. Cipolla et al. [154] found that rhDNase following nebulization in all four devices evaluated (T Up-Draft II, customized Respirgard II, Acorn II, and Airlife Misty) resulted in no loss of enzymatic activity and no increase in aggregation. Circular dichroism spectrophotometry indicated no change in either the secondary or tertiary structure in rhDNase following nebulization.

5. Selecting an Optimal Site of Absorption in the Gastrointestinal Tract

To maximize the probability of achieving therapeutically significant bioavailability from oral administration, it seems prudent to consider the concept of targeting drug release in a region of the gastrointestinal tract that favors absorption for reasons of high permeability and/or low proteolytic activity. On the basis of permeability, the ileum appears to be the best site to administer insulin [156]. However, protection from proteolysis is required to achieve significant bioavailability. This hypothesis has been confirmed in a study by Morishita et al. [157], using microspheres designed to release insulin in different parts of the small intestine. It is doubtful that microsphere-entrapped insulin gained access to the systemic circulation by way of the Peyer's patches, although polystyrene microspheres, 0.46 mm in diameter, are known to be captured by the overlying M cells at a rate of 10^5 microspheres/45 min [158]. This is because drugs captured by the M cells in the Peyer's

patches do not have direct access to the systemic circulation, only to the lymphatic circulation. In addition to microspheres, li-

posomes larger than 0.35 mm in diameter that can survive the harsh environment of the gastrointestinal tract are also preferentially endocytized by the M cells overlying the Peyer's patches [159]. Uptake of microparticulates may be enhanced by exploiting the unique glycoconjugates that may exist on the M cells, such as fucose and N-acetylgalactosamine [160]. Proteinoid microspheres encapsulating influenza virus antigens are an attractive but unproven vehicle for oral immunization [161].

The latest documented attempt to target the delivery of insulin orally at a specific site in the gastrointestinal tract is that of Ziv et al. [162]. These investigators administered insulin (5 mg) to both diabetic and nondiabetic dogs in microtablets, having a diameter of 0.61.0 mm, that were enteric-coated with 1020% Eudragit L-100 and loaded with 123 mg/g of sodium cholate and 40 mg/g of soybean trypsin inhibitor. There was a progressive fall in blood glucose, reaching a minimum of 2040% of the initial values at 60140 min. Hypoglycemia lasted for about 90 min. Although the bioavailability was not determined, the variability in peak response was encouraging minimal.

During the past 10 years, the colon has sporadically received attention as a potential site for the delivery of peptide and protein drugs due to its low proteolytic activities [163] and long residence time of 1020 h, on average [164]. Studies on mucus gel thickness and turnover in the gastrointestinal tract of the rat by Rubinstein and Tirosh [165] suggest that the colon is preferred to the upper intestine as the location for mucoadhesion. This is because the mucus turnover rate is slower, its sensitivity to mucus secretory stimulus is lower, and the adhesion force between mucus and polycarbophil (the mucoadhesive) is stronger.

A formidable barrier to absorption from the colon is its low permeability when compared with the upper small intestine. A case in point is the absorption of benazepril, a new oral nonsulfhydryl selective angiotensin-converting enzyme (ACE) inhibitor. Chan et al. [166] found that, in humans, the colon delivered 23% whereas the small intestine delivered 90% of the oral dose into the systemic circulation. In addition, the absorption rate from the colon was three times slower than from the small intestine. Although the permeability in the upper small intestine was not measured, the permeability coefficient of IGF-I (m.w. 7649) in the colon was found to be $8.03 \pm 1.03 \times 10^{-8}$ cm/s in the rat and $4.75 \pm 0.43 \times 10^{-8}$ cm/s in the minipig [167]. Such a permeability would potentially provide a clinically significant amount of insulin growth factor-I (IGF-I) in the systemic circulation, provided that the entire length of the colon is available for absorption and that IGF-I is not degraded by the microbial proteases in the lumen or adsorbed onto the mucosal cells [168]. A potential solution to the low permeability problem is to coadminister the peptide with penetration enhancers. Indeed, Morishita et al. [156] reported that the rat colon was

more sensitive to the effect of penetration enhancers than the small intestine.

Regardless of the ultimate site of preferred peptide absorption in the gastro-intestinal tract, clearance of the absorbed drug, including biliary excretion, by the liver [169,170] cannot be overlooked as a barrier to oral peptide and protein drug delivery. In fact, knowledge of such processes is limited. The pseudohexapeptide renin inhibitor ditekiren is an example of a peptide that is extensively eliminated by biliary excretion following intravenous administration [171,172]. Another example is fosinoprilat, an ACE inhibitor that is excreted into bile to the extent of 46% [37]. Using the isolated perfused rat liver technique, Adedoyin et al. [173] observed dose dependency in the uptake of ditekiren by hepatocytes as well as elimination into bile, more so for the latter than the former. It was proposed that a different carrier was involved in each step. Although the carrier responsible for canalicular peptide transport has not been identified, a possible candidate in the hepatocyte uptake process is the multispecific bile acid carrier of the hepatic basolateral membrane [174,175]. Other candidate carriers are those for organic anions [176] and organic cations [177]. The future design of peptide drugs with good systemic bioavailability must take the potentially negative influence of biliary clearance into account.

III. Preabsorptive Metabolic Clearance of Peptide and Protein Drugs

Metabolism of peptide and protein drugs by proteases in the lumen and mucosal cells, thereby limiting their absorption, is well known [68,178]. Metabolism at the site of intramuscular and subcutaneous injection is often overlooked as a factor limiting systemic peptide entry. This, in fact, explains the relatively low bioavailability (24%) of the ACTH(124) fragment, tetracosactide, in the rat [179].

Studies by Lee and his co-workers [180182] reveal that protease activities in the homogenates of the nasal, buccal, rectal, and vaginal mucosae of the albino rabbit are substantial and, in fact, are comparable to those in the ileal homogenate. This partly explains the less than complete bioavailability of peptides and proteins administered via the nonparenteral routes. Methionine enkephalin was three and nine times more susceptible to degradation in the mucosal extracts of the vagina and the nose of the rabbit, respectively, than in the mucosal extract of the rectum [183]. It was surprising that the metabolic barrier in the alveolar epithelium appears to be about five times lower than that in other mucosal epithelia [184]. Differences in the extent of proteolytic destruction between the oral and pulmonary routes of administration possibly explain the 0.5% bioavailability of an RGD tetrapeptide from intraduodenal administration [185] when compared with the 20% bioavailability from intratracheal administration [143]. Both aminopeptidases and dipeptidyl carboxypeptidase were present in the alveolar cells to degrade YGGFM, while dipeptidyl peptidase contributed

much less to the degradation. Nevertheless, Gly-L-Phe was substantially metabolized during transport across cultured alveolar epithelial monolayers [186]. The proportion of L-Phe, a metabolite, observed at 4 h in the basolateral receiver fluid for 1 and 10 mM apical donor Gly-L-Phe accounted for 83% and 77% of the estimated total Gly-L-Phe, respectively.

Aprotinin and, to a lesser extent, soybean trypsin inhibitor [187], have been tested for their potency in enhancing peptide and protein absorption through protease inhibition [156]. A newcomer is bacitracin. Wüthrich et al. [179] reported that 0.01 M bacitracin enhanced the nasal absorption of tetracosactide in the rat from 4 to 24%, compared with 12% afforded by 1% Na glycocholate. The coadministration of 2 mM puromycin, an aminopeptidase inhibitor, in a pH 4 formulation with metkephamid (Tyr-D-Ala-Gly-Phe-N-Me-Met2) improved its oral bioavailability in the rat from 0.22 ± 0.06% to 4.5 ± 1.7% [188]. Various aminopeptidase inhibitors with greater potency than bestatin, amastatin, and puromycin have been developed to protect peptides against degradation, including a phosphinic acid dipeptide analog [189] and aminoboronic acid derivatives [190]. Thus far, they have been tested against proteases in the nasal cavity, but their efficacy in enhancing systemic peptide bioavailability has not yet been revealed. As in the case with penetration enhancers, it is imperative that the effect of these aminopeptidase inhibitors on membrane integrity be tested [191].

IV. Systemic Clearance of Peptide and Protein Drugs.

The plasma half-life of natural peptides and proteins is relatively short. It is less than 11 min for recombinant human transforming growth factor (TGF-b1) [192] and 2030 min for two neurotensin hexapeptides [193], both in the rat. Rapid clearance may be mediated by clearance receptors, as is the case for atrial natriuretic factor [194], epidermal growth factor (EGF) [195], and insulin [196]; by proteolytic degradation, as is the case for neurotensin [193], atrial natriuretic factor [194], and many others; or by glomerular filtration, as in the case for cytokines [197] and FITC-dextrans [198].

A. Role of Liver and Kidney

The liver and kidney play a central role in clearing peptide and protein drugs from the bloodstream. The liver plays a major role in the clearance of C-terminal and N-terminal atrial natriuretic factor in the rat [199]. The hepatic extraction ratio is 41 ± 5% for the C-terminal moiety and 39 ± 6% for the N-terminal moiety. Other peptides and proteins for which the liver plays a key role in their clearance are EGF [195], insulin [196], and many others.

The kidney plays a key role in the clearance of recombinant human interferon-aA in

rabbits [200]. No dose-dependent clearance is seen over a 1000-

fold range of doses (i.e., 10^6-10^9 U kg^{-1}). Kim et al. [201] demonstrated that the EGF receptor on the antiluminal side of the nephron was involved, at least in part, in the renal clearance of EGF. Filtered EGF was reabsorbed through low-affinity binding sites on the luminal cell surface membrane [201]; so was superoxide dismutase (SOD, m.w. 32,000), albeit via a different mechanism [202]. A nonreceptor uptake mechanism possibly exists on the luminal side to extract cationic macromolecules [203]. Erythropoietin, which normally is not cleared by the kidney, suffered extensive urinary loss in nephrotic rats plagued with a significant reduction in hematocrit, hemoglobin, and erythrocyte count. The net result was a doubling of the systemic clearance of erythropoietin [204].

B. Approaches to Reduce Systemic Clearance

The approaches to reduce systemic clearance can be classified into two categories: chemical modification and incorporation into sustained drug delivery systems.

1. Chemical Modification

The common denominator in approaches based on chemical modification is to reduce hepatic and/or renal clearance. The best known approach is that based on conjugation of a given protein with water-soluble polymers, notably PEG. Thus, the conjugation of a_1-proteinase inhibitor (m.w. 52K) with PEG-4000 greatly slows its clearance by the kidney without affecting its metabolism and recognition by the receptor [205]. The extension in plasma half-life is a function of the PEG's molecular weight [206]; the increase in half-life is from 3.5 min to 9 or more hours. Similar favorable results have been obtained for lactoferrin and a_2-macroglobulin-trypsin complex [206]. It is conceivable that further gain in prolongation of plasma half-life would be achieved by anionizing the protein molecule. Using the perfused rat kidney, Mihara et al. [203] found that cationic DEAE-dextrans were excreted more extensively than anionic ones.

An alternative strategy to PEG conjugation, first tested in SOD, is to link covalently the lysine residues of a protein with poly(styrene-co-maleic acid)butyl ester, resulting in a derivative that reversibly binds to albumin. Such a construct extended the half-life of SOD from 4 min to 6 h in the rat [207].

A fundamentally different approach is exemplified in the lipophilic derivatization of low molecular weight heparin (m.w. 5800) to yield longer-lasting inhibitory effects on the coagulation system [208]. This is apparently due to binding of the derivatized heparin to serum albumin and reduced renal excretion. There is as yet another approach based on modification of the terminal sugar of a glycoprotein, especially when the terminal sugar rate-limits its clearance from the systemic circulation. Thus, Nishikawa et al. [209] observed that galactosylated and glycosylated BSA are cleared more rapidly than

mannosylated BSA,

consistent with the relative distribution of galactose and glucose receptors in the parenchymal cells vs. mannose receptors in the nonparenchymal cells.

2. Incorporation in Sustained Drug Delivery Systems

Several systems have been tested for their ability to sustain the release of peptides and proteins, such as insulin, vasopressin, oxytocin, and luteinizing hormone-releasing hormone. These systems include nondegradable [210217] and degradable polymeric systems [218,219], liposomes [220224], and osmotic pumps [225227]. Sustained drug delivery for up to 30 days has been observed for thyrotropin-releasing hormone following subcutaneous administration in poly(lactide-co-glycolide) (PLGA) microspheres in rats [228]. Encapsulation of interleukin-7 (IL-7), a 25 kD cytokine originally derived from the stromal element of a bone marrow cell line, in a unilamellar liposomal suspension comprising egg phosphatidylcholine and cholesterol (8:2 w/w) substantially prolonged the half-life of IL-7 in blood from 116 to 3018 min in the guinea pig, but at the expense of a reduced peak concentration to less than one-tenth of that afforded by soluble IL-7. There was, however, no change in the bioavailability. A reduced rate of IL-7 disappearance from the injection site in the liposomal formulation was responsible for the sustained blood level of IL-7. The time course of blood lymphocyte numbers was in excellent agreement with the IL-7 concentration-time course.

A noteworthy recent development potentially useful in sustaining peptide and protein drug delivery following intravenous (i.v.) administration is that of Gref et al. [229]. These investigators discovered that biodegradable nanospheres (140 ± 10 nm in diameter) made of amphiphilic copolymers composed of PEG and poly(lactic-glycolic acid) or poly(caprolactone) exhibited dramatically increased blood circulation times and reduced liver accumulation in mice. This approach is an extension of an earlier one based on the incorporation of monosialoganglioside (GM_1) or polyethylene glycol conjugated to phosphatidylethanolamine in the bilayers of large unilamellar liposomes [161].

There are two concerns with the use of sustained drug delivery systems to prolong the plasma concentration of peptide and protein drugs. First, there is the risk of inducing an immune response to the protein entrapped in a polymeric matrix. Uchida et al. [230] reported that ovalbumin loaded to the extent of 0.05%, 0.1%, 0.5%, and 1% in biodegradable poly(lactide-co-glycolide) microspheres elicited a systemic immune response following subcutaneous administration in Balb C mice 68 weeks of age. A similar response was observed upon the administration of high doses of granulocyte-macrophage colony-stimulating factor encapsulated in cell-sized gelatin chondroitin sulfate microspheres [231].

The second concern is that a zero-order pattern of drug release may not be optimal for peptide and protein drugs, particularly for those with peculiar dose-

response curves. A case in point is luteinizing hormone-releasing hormone. The agonistic activity of this peptide hormone would be manifest only when delivered in a pulsatile fashion; otherwise, antagonistic activity would result [232]. Growth hormone is another protein drug that exhibits a more intense pharmacodynamic response when given in pulses rather than in a bolus [233]. Several non-zero drug delivery systems have been investigated [234], the most sophisticated of which is the self-regulating system [235237]. The construct of such a delivery system depends on two key elements: a trigger, which often is a marker characteristic of the disease state; and a system property that would recognize the trigger, which can either be the physicochemical properties of the peptide and protein drug (e.g., solubility) or the physicochemical properties of the polymer forming the system (e.g., porosity and erodibility). Of these two elements, the trigger is often the more elusive, since it may not be as obvious as is the case in diabetes mellitus. It is therefore reasonable to state that success in the design of self-regulating drug delivery systems requires a close alliance between pathophysiologists and polymer chemists.

V. Conclusion

Peptide and protein drugs are not simply a passing fancy in drug delivery. They are here to stay, and will grow in importance. The last decade has witnessed an expansion of efforts to refine existing drug delivery systems and create new ones with the common goal of making peptide and protein drugs a therapeutic reality, particularly in the treatment of diseases that have thus far eluded the most powerful medicines known. We are in the midst of an intense race towards the creation of imaginative drug delivery strategies that will benefit not only peptide and protein drugs but also nonpeptide and protein drugs. This goal can only be fulfilled through a thorough understanding of the biochemistry, biophysics, and physiology of the drug's administration and target sites.

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References

1. A. Kass-Eisler, E. Falck-Pedersen, M. Alvira, J. Rivera, P. M. Buttrick, B. A. Wittenberg, L. Cipriani, and L. A. Leinwand. Proc. Natl. Acad. Sci. USA 90:11498, 1993.

2. N. Murahashi, A. Kato, N. Koyama, S. Watanabe, T. Yuzuriha, and Y. Miyaki. J. Pharm. Pharmacol. 41:770, 1989.

3. H. Okada, I. Yamazaki, Y. Ogawa, S. Hirai, T. Yashiki, and H. Mima. J. Pharm. Sci. 71:1367, 1982.

4. K. Morimoto, H. Akatsuchi, R. Aikawa, M. Morishita, and K. Monsaki. J. Pharm. Sci. 73:1366, 1984.

5. G. Golomb, A. Avramoff, and A. Hoffman. Pharm. Res. 10:828, 1993.

6. A. Allen, A. Garner, D. Hutton, and S. McQueen. J. Physiol. 341:66, 1983.

7. T. Sakata and M. Engelhardt. Cell Tissue Res. 219:629, 1981.

8. T. Shimada. J. Physiol. 392:113, 1987.

9. P. A. Edwards. Br. Med. Bull. 34:55, 1978.

10. D. Winne and W. Verheyen. J. Pharm. Pharmacol. 42:507, 1990.

11. A. Wikman, J. Karlsson, I. Carlstedt, and P. Artursson. Pharm. Res. 10:843, 1993.

12. L. Hovgaard, E. J. Mack, and S. W. Kim. J. Contr. Rel. 19:99, 1992.

13. B. M. Gumbiner. J. Cell Biol. 123:1631, 1993.

14. J. R. Pappenheimer. J. Membr. Biol. 100:137, 1987.

15. P. Artursson, A. L. Ungell, and J. E. Lofroth. Pharm. Res. 10:1123, 1993.

16. D. C. Corbo, J. C. Liu, and Y. W. Chien. J. Pharm. Sci. 79:202, 1990.

17. K. Hosoya, H. Kubo, H. Natsume, K. Sugibayashi, Y. Mirimoto, and S. Yamashita. Biopharm. Drug Disp. 14:685, 1993.

18. Y. Rojanasakul, L. Y. Wang, M. Bhat, D. D. Glover, C. J. Malanga, and J. K. H. Ma. Pharm. Res. 9:1029, 1992.

19. A. J. Hoogstraate, C. Cullander, J. F. Nagelkerke, S. Senel, J. C. Verhoef, H. E. Junginger, and H. E. Bodde. Pharm. Res. 11:83, 1994.

20. U. Jaehde, R. Masereeuw, A. G. DeBoer, G. Fricker, J. F. Nagelkerke, J. Vonderscher, and D. D. Breimer. Pharm. Res. 11:442, 1994.

21. C. McMartin, L. E. F. Hutchinson, R. Hyde, and G. E. Peters. J. Pharm. Sci. 76:535, 1987.

22. H. Kubo, K. Hosoya, H. Natsume, K. Sugibayashi, and Y. Morimoto. Int. J. Pharm. 103:27, 1994.

23. A. Yamamoto, T. Morita, M. Hashida, and H. Sezaki. Int. J. Pharm. 93:91, 1993.

24. K. H. Soergel. Gastroenterology 105:1247, 1993.

25. W. C. Yen and V. H. L. Lee. J. Control. Release 28:97, 1994.

26. E. Wunsch and H. G. Heidrch. Hoppe-Seylers Z. Physiol. Chem. 332:300, 1963.

27. S. G. Schultz, R. A. Frizzell, and H. N. Nellans. Ann. Rev. Physiol. 36:51, 1974.

28. M. A. Wheatley, J. Dent, E. B. Wheeldon, and P. L. Smith. J. Control. Release 8:167, 1988.

29. J. R. Pappenheimer and K. Z. Reiss. J. Membr. Biol. 100:123, 1987.

30. K. Atisook and J. L. Madara. Gastroenterology 100:719, 1991.

31. W. Clauss, H. Schafer, I. Horch, and H. Hornicke. Pflugers Arch. 403:278, 1985.

32. K. Ewe. Pharmacology 36:73, 1988.

33. M. S. Mooseker, T. A. Graves, K. A. Wharton, N. Falco, and C. L. Howe. J. Cell Biol. 87:809, 1980.

34. J. Llopis, G. E. N. Kass, S. K. Duddy, G. A. Moore, and S. Orrenius. FEBS Lett. 280:84, 1991.

35. R. A. Conradi, A. R. Hilgers, N. F. H. Ho, and P. S. Burton. Pharm. Res. 8:1453, 1991.

36. E. G. Chikhale, K. Y. Ng, P. S. Burton, and R. T. Borchardt. Pharm. Res. 11:412, 1994.

37. S. A. Ranadive, A. X. Chen, and A. T. M. Serajuddin. Pharm. Res. 9:1480, 1992.

38. F. J. Sharom, X. Yu, and C. A. Doige, J. Biol. Chem. 268:24197, 1993.

39. V. Ling. Cancer 69:2603, 1992.

40. S. Hsing, Z. Gatmaitan, and I. M. Arias. Gastroenterology 102:879, 1992.

41. P. F. Augustijns, T. P. Bradshaw, L. S. L. Gan, R. W. Hendren, and D. R. Thakker. Biochem. Biophys. Res. Commun. 197:360, 1993.

42. J. Hunter, B. H. Hirst, and N. L. Simmons. Pharm. Res. 10:743, 1993.

43. P. S. Burton, R. A. Conradi, A. R. Hilgers, and N. F. H. Ho. Biochem. Biophys. Res. Commun. 190:760, 1993.

44. K. Morimoto, H. Yamahara, and V. H. L. Lee. Pharm. Res. 10:1668, 1993.

45. A. Tsuji, T. Terasaki, I. Tamai, and H. Hirooks. J. Pharmacol. Exp. Ther. 241:594, 1987.

46. P. J. Sinko and G. L. Amidon. J. Pharm. Sci. 78:723, 1989.

47. D. I. Friedman and J. W. Payne. Pharm. Res. 6:1043, 1989.

48. D. I. Friedman and G. L. Amidon. J. Pharm. Sci. 78:995, 1989.

49. H. Daniel, E. L. Morse, and S. A. Adibi. J. Biol. Chem. 266:19917, 1991.

50. P. F. Bai, P. Subramanian, H. I. Mosberg, and G. L. Amidon. Pharm. Res. 8:593, 1991.

51. K. Iseki, K. I. Mori, K. Miyazaki, and T. Arita. Biochem. Pharmacol. 36:1837, 1987.

52. K. Iseki, K. I. Mori, K. Miyazaki, and T. Arita. Biochem. Pharmacol. 36:1843, 1987.

53. W. Kramer, F. Girbig, I. Leipe, and E. Petzoldt. Biochem. Pharmacol. 37:2427, 1988.

54. Y. Miyamoto, Y. G. Thompson, E. F. Howard, V. Ganapathy, and F. H. Leibach. J. Biol. Chem. 266:4742, 1991.

55. Y. J. Fel, Y. Kanal, S. Nussberger, V. Ganapathy, F. H. Lelbach, M.F. Romero, S. K. Singh, W. F. Boron, and M.A. Hediger. Nature 368:563, 1994.

56. J. Dyer, R. B. Beechey, J. P. Gorvel, R. T. Smith, T. Wootton, and S. P. Shirazi-Beechey. Biochem. J. 269:565, 1990.

57. K. Inui, M. Yamamoto, and H. Saito. J. Pharmacol. Exp. Ther. 261:195, 1992.

58. D. T. Thwaites, C. D. A. Brown, B. H. Hirst, and N. L. Simmons. J. Biol. Chem. 268:7640, 1993.

59. W. C. Shen, J. Wan, and H. Ekrami. Adv. Drug Deliv. Rev. 8:93, 1992.

60. E. E. Wheeler, D. N. Challacombe, P. J. Kerry, and E. C. Pearson. J. Pediatr. Gastroenterol. Nutr. 16:157, 1993.

61. M. Heyman, A. M. Crain-Denoyelle, and J. F. Desjeux. J. Pediatr. Gastroenterol. Nutr. 9:238, 1989.

62. K. Siminoski, P. Gonnella, J. Bernanke, L. Owen, M. Neutra, and R. A. Murphy. J. Cell. Biol. 103:1979, 1986.

63. R. W. Jansen, P. Olinga, G. Harms, and D. K. F. Meijer. Pharm. Res. 10:1611, 1993.

64. H. Tokuda, S. Masuda, Y. Takakura, H. Sezaki, and M. Hashida. Biochem. Biophys. Res. Commun. 196:18, 1993.

65. I. Toth. In Drug Absorption Enhancement. Concepts, Possibilities, Limitations and Trends. Edited by A. G. de Boer. Harwood Academic, Netherlands, 1994, p 249.

66. A. H. Kahns, A. Buur, and H. Bundgaard. Pharm. Res. 10:68, 1993.

67. S. Muranishi, M. Murakami, M. Hashidzume, K. Yamada, S. Tajima, and Y. Kiso. J. Control. Release 19:179, 1992.

68. V. H. L. Lee and A. Yamamoto. Adv. Drug Deliv. Rev. 4:171, 1990.

69. L. Narawane and V. H. L. Lee. In Drug Absorption Enhancement. Concepts, Possibilities, Limitations and Trends. Edited by A. G. de Boer. Harwood Academic, Netherlands, 1994, p 1.

70. S. Hirai, T. Yashiki, and H. Mima. Int. J. Pharm. 9:173, 1981.

71. G. S. Gordon, A. C. Moses, R. D. Silver, J. S. Flier, and M. C. Carey. Proc. Natl. Acad. Sci. USA 82:7419, 1985.

72. A. Yamamoto, E. Hayakawa, Y. Kato, A. Nishiura, and V. H. L. Lee. J. Pharmacol. Exp. Ther. 263:25, 1992.

73. S. Yoshioka, L. Caldwell, and T. Higuchi. J. Pharm. Sci. 71:593, 1982.

74. A. C. Moses, G. S. Gordon, M. C. Carey, and J. S. Flier. Diabetes 32:1040, 1983.

75. R. Salzman, J. E. Manson, G. T. Griffing, R. Kimmerle, N. Small, J. Armstrong, and J. C. Melby. N. Engl. J. Med. 312:1078, 1985.

76. T. Nishihata, J. H. Rytting, A. Kamada, T. Higuchi, M. Routh, and L. Caldwell. J. Pharm. Pharmacol. 35:148, 1983.

77. T. Nishihata, C. S. Lee, M. Yamamoto, J. H. Rytting, and T. Higuchi. J. Pharm. Sci. 73:1326, 1984.

78. A. Kamada, T. Nishihata, S. Kim, M. Yamamoto, and N. Yata. Chem. Pharm. Bull. 29:2012, 1981.

79. T. Nishihata, Y. Okamura, A. Kamada, T. Higuchi, T. Yagi, R. Kawamori, and M. Shichiri. J. Pharm. Pharmacol. 37:22, 1985.

80. P. M. Reardon, D. A. Wall, T. K. Hart, P. L. Smith, and C. H. Gochoco. Pharm. Res. 10:1301, 1993.

81. W. A. Lee, B. A. Narog, T. W. Patapoff, and Y. J. Wang. J. Pharm. Sci. 80:725, 1991.

82. J. P. Longenecker, A. C. Moses, J. S. Flier, R. D. Silver, M. C. Carey, and E. J. Dubovi. J. Pharm. Sci. 76:351, 1987.

83. E. J. van Hoogdalem, C. D. Heijligers-Feijen, R. A. A. Mathot, A. T. E. Wackwitz, J. B. M. M. van Bree, J. C. Verhoeff, A. G. DeBoer, and D. D. Breimer. J. Pharmacol. Exp. Ther. 251:741, 1989.

84. S. Lundin, N. Pantzer, L. Hedin, and B. R. Weström. Int. J. Pharm. 59:263, 1990.

85. Y. Watanabe, K. Matsumoto, K. Kawamoto, S. Yazawa, and M. Matsumoto. Chem. Pharm. Bull. 40:3100, 1992.

86. Y. Watanabe, Y. Matsumoto, M. Seki, M. Takase, and M. Matsumoto. Chem. Pharm. Bull. 40:3042, 1992.

87. J. A. Fix, K. Engle, P. A. Porter, P. S. Leppert, S. J. Selk, C. R. Gardner, and J. Alexander. Am. J. Physiol. 251:G332, 1986.

88. K. Takahashi, T. Murakami, A. Kamata, R. Yumoto, Y. Higashi, and N. Yata. Pharm. Res. 11:388, 1994.

89. Y. Watanabe, Y. Matsumoto, N. Hori, H. Funato, and M. Matsumoto. Chem. Pharm. Bull. 39:3007, 1991.

90. L. Lohikangas, M. Wilen, M. Einarsson, and P. Artursson. Eur. J. Pharm. Sci. 1:297, 1994.

91. I. J. Gill, A. N. Fisher, M. Hinchcliffe, J. Whitestone, N. Farraj, R. De Ponti, and L. Illum. Eur. J. Pharm. Sci. 1:237, 1994.

92. E. Hayakawa, A. Yamamoto, Y. Shoji, and V. H. L. Lee. Life Sci. 45:167, 1989.

93. Y. Li, Z. Shao, and A.K. Mitra. Pharm. Res. 9:864, 1992.

94. H.-H. Lu, J. D. Thomas, J. J. Tukker, and D. Fleisher. Pharm. Res. 9:894, 1992.

95. N. A. See and P. Bass. J. Pharm. Sci. 82:721, 1993.

96. P. Midoux, C. Mendes, A. Legrand, J. Raimond, R. Mayer, M. Monsigny, and A. C. Roche. Nucleic Acids Res. 21:871, 1993.

97. J. C. Villegas and R. D. Broadwell. J. Neurocytol. 22:67, 1993.

98. C. M. Lehr and V. H. L. Lee. Pharm. Res. 10:1796, 1993.

99. C. Ropert, C. Malvy, and P. Couvreur. Pharm. Res. 10:1427, 1994.

100. T. Murakami, H. Kawakita, M. Kishimoto, Y. Higashi, T. Amagase, T. Hayashi, N. Nojima, T. Fuwa, and N. Yata. Int. J. Pharm. 46:9, 1988.

101. T. Sawada, T. Ogawa, M. Tomita, M. Hayashi, and S. Awazu. Pharm. Res. 8:1365, 1991.

102. E. K. Anderberg, T. Lindmark, and P. Artursson. Pharm. Res. 10:857, 1993.

103. J. Hastewell, S. Lynch, R. Fox, I. Williamson, P. Skelton-Stround, and M. Mackay. Int. J. Pharm. 101:115, 1994.

104. W. F. Stenson, R. A. Easom, T. E. Riehl, and J. Turk. Am. J. Physiol. 265:G955, 1993.

105. M. S. Balda, L. Gonzalez-Mariscal, K. Matter, and M. Cereijido. J. Cell Biol. 123:293, 1993.

106. W. A. Lee, R. D. Ennis, J. P. Longenecker, and P. Bengtsson. Pharm. Res. 11:747, 1994.

107. J. Drewe, G. Fricker, J. Vonderscher, and C. Beglinger. Br. J. Pharmacol. 108:298,

1993.

108. L. Lohikangas, M. Wilen, M. Einarsson, and P. Artursson. Eur. J. Pharm. Sci. 1:307, 1994.

109. K. Hosoya, H. Kubo, H. Natsume, K. Sugibayashi, and Y. Morimoto. Biol. Pharm. Bull. 17:316, 1994.

110. M. D. Donovan, G. L. Flynn, and G. L. Amidon. Pharm. Res. 7:863, 1990.

111. D. T. O'Hagan, H. Critchley, N. F. Farraj, A. N. Fisher, B. R. Johansen, S.S. Davis, and L. Illum. Pharm. Res. 7:772, 1990.

112. N. A. Flavahan. Am. J. Physiol. 264:H722, 1993.

113. I. J. Gill, L. Illum, N. Farraj, and R. De Ponti. Eur. J. Pharm. Sci. 1:229, 1994.

114. T. Kissel, J. Drewe, S. Bantle, A. Rummelt, and C. Beglinger. Pharm. Res. 9:52, 1992.

115. W. A. Lee, H. F. L. Lu, P. W. Maffuid, M. T. Botet, P. A. Baldwin, T. A. Benkert, and C. K. Klingbeil. J. Control. Release 22:223, 1992.

116. K. Kontturi, L. Murtomaki, J. Hirvonen, P. Paronen, and A. Urtti. Pharm. Res. 10:381, 1993.

117. S. M. Sims, W. I. Higuchi, and V. Srinivasan. Pharm. Res. 9:1402, 1992.

118. R. R. Burnett and D. Marreo. J. Pharm. Sci. 75:738, 1986.

119. M. C. Heit, P. L. Williams, F. L. Jayes, S. K. Chang, and J. E. Riviere. J. Pharm. Sci. 82:240, 1993.

120. S. Kumar, H. Char, S. Patel, D. Piemontese, K. Iqbal, A. W. Malick, E. Neugroschel, and C. R. Behl. J. Pharm. Sci. 81:635, 1992.

121. B. Kari. Diabetes 35:217, 1986.

122. O. Siddiqui, Y. Sun, J. C. Liu, and Y. W. Chien. J. Pharm. Sci. 76:341, 1987.

123. P. Lelawongs, J. C. Liu, O. Siddiqui, and Y. W. Chien. Int. J. Pharm. 56:13, 1989.

124. B. H. Sage, Jr., and J. E. Riviere. Adv. Drug Deliv. Rev. 9:265, 1992.

125. C. Cullander. Adv. Drug Deliv. Rev. 9:119, 1992.

126. N. A. Monteiro-Riviere, A. O. Inman, and J. E. Riviere. Pharm. Res. 11:251, 1994.

127. M. J. Pikal and S. Shah. Pharm. Res. 7:213, 1990.

128. M. J. Pikal. Adv. Drug Deliv. Rev. 9:201, 1992.

129. M. B. Delgado-Charro and R. H. Guy. Pharm. Res. 11:929, 1994.

130. H. Inada, A. H. Ghanem, and W. I. Higuchi. Pharm. Res. 11:687, 1994.

131. M. C. Heit, N. A. Monteiro-Riviere, F. L. Jayes, and J. E. Riviere. Pharm. Res. 11:1000, 1994.

132. E. A. Mueller, J. M. Kovarik, J. B. van Bree, W. Tetzloff, J. Grevel, and K. Kutz. Pharm. Res. 11:301, 1994.

133. R. J. Scothorne. In Cunningham's Textbook of Anatomy. Edited by G. J. Romanes. University Press, Oxford, 1987, p 491.

134. R. W. Niven and P. R. Byron. Pharm. Res. 7:8, 1990.

135. R. W. Niven, F. Rypacek, and P. R. Byron. Pharm. Res. 7:990, 1990.

136. J. S. Patton and R. M. Platz. Adv. Drug. Deliv. Rev. 8:179, 1992.

137. F. Y. Liu, D. O. Kildsig, and A. K. Mitra. Life Sci. 51:1683, 1992.

138. P. R. Byron, Z. Sun, H. Katayama, and F. Rypacek. Pharm. Res. 11:221, 1994.

139. P. Colthorpe, S. J. Farr, G. Taylor, I. Smith, and D. Wyatt. Pharm. Res. 9:764, 1992.

140. Y. Watanabe, Y. Matsumoto, M. Yamagushi, R. Kikuchi, K. Takayama, H. Nomura, K. Maruyama, and M. Matsumoto. Biol. Pharm. Bull. 16:93, 1993.

141. M. Machida, K. Sano, M. Arakawa, M. Hayashi, and S. Awazu. Pharm. Res. 10:1372, 1993.

142. K. Okumura, S. Iwakawa, T. Yoshida, T. Seki, and F. Komada. Int. J. Pharm. 88:63, 1992.

143. P. L. Smith, J. Marcello, D. C. Chiossone, D. Orner, and I. J. Hidalgo. Int. J. Pharm. 106:95, 1994.

144. D. B. Bennett, E. Tyson, C. A. Nerenberg, S. Mah, J. S. deGroot, and Z. Teitelbaum. Pharm. Res. 11:1048, 1994.

145. H. Schreier, K. J. McNicol, D. B. Bennett, Z. Teitelbaum, and H. Derendorf. Pharm. Res. 11:1056, 1994.

146. T. B. Martonen and I. M. Katz. Pharm. Res. 10:871, 1993.

147. F. Komada, S. Iwakawa, N. Yamamoto, H. Sakakibara, and K. Okumura. J. Pharm. Sci. 83:763, 1994.

148. L. E. Masinde and A. J. Hickey. Int. J. Pharm. 100:123, 1993.

149. K. Morimoto, H. Yamahara, V. H. L. Lee, and K. J. Kim. Life Sci. 54:2083, 1994.

150. Y. Li, Z. Shao, D. B. DeNicola, and A. K. Mitra. Eur. J. Pharm. Biopharm. 39:216, 1993.

151. L. Y. Wang, J. K. H. Ma, W. F. Pan, D. Toledo-Velasquez, C.J. Malanga, and Y. Rojanasakul. Pharm. Res. 11:513, 1994.

152. P. R. Byron. Adv. Drug Deliv. Rev. 5:107, 1990.

153. Y. Sato. J. Aerospace Med. 5:59, 1992.

154. D. Cipolla, I. Gonda, and S. J. Shire. Pharm. Res. 11:491, 1994.

155. R. C. Hubbard, N. G. McElvaney, P. Birrer, S. Shak, W. W. Robinson, C. J. Margaret, M. S. Chernick, and R. G. Crystal. N. Engl. J. Med. 326:812, 1992.

156. M. Morishita, I. Morishita, K. Takayama, Y. Machida, and T. Nagai. Biol. Pharm. Bull. 16:68, 1993.

157. I. Morishita, M. Morishita, K. Takayama, Y. Machida, and T. Nagai. Int. J. Pharm. 91:29, 1993.

158. M. A. Jepson, N. L. Simmons, T. C. Savidge, P. S. James, and B. H. Hirst. Cell Tissue Res. 271:399, 1993.

159. Y. Aramaki, H. Tomizawa, T. Hara, K. Yachi, H. Kikuch, and S. Tsuchiya. Pharm. Res. 10:1228, 1993.

160. A. Gebert and G. Hach. Gastroenterology 105:1350, 1993.

161. N. Santiago, S. Milstein, T. Rivera, E. Garcia, T. Zaidi, H. Hong, and D. Bucher. Pharm. Res. 10:1243, 1993.

162. E. Ziv, M. Kidron, I. Raz, M. Krausz, Y. Blatt, A. Rotman, and H. Bar-On. J. Pharm. Sci. 83:792, 1994.

163. J. P. F. Bai. Life Sci. 52:941, 1993.

164. B. Krevsky, L. S. Malmud, F. D'Ercole, A. H. Maurer, and R. S. Fisher. Gastroenterology 91:1102, 1986.

165. A. Rubinstein and B. Tirosh. Pharm. Res. 11:794, 1994.

166. K. K. H. Chan, A. Buch, R. D. Glazer, V. A. John, and W. H. Barr. Pharm. Res.

11:432, 1994.

167. E. Quadros, N. M. Landzert, S. LeRoy, F. Gasparini, and G. Worosila. Pharm. Res. 11:226, 1994.

168. G. T. McFarlane, C. Allison, S. A. W. Gibson, and J. H. Cummings. J. Appl. Bacteriol. 64:37, 1988.

169. K. Ziegler, M. Frimmer, H. Kessler, I. Damm, V. Eirmann, S. Koll, and J. Zarbock. Biochem. Biophys. Acta 845:86, 1985.

170. K. Ziegler, W. Lins, and M. Frimmer. Biochim. Biophys. Acta 1061:287, 1991.

171. J. C. Greenfield, K. J. Cook, and I. A. O'Leary. Drug Metab. Dispos. 17:518, 1989.

172. B. D. Rush, K. F. Wilkinson, W. Z. Zhong, S. K. Closson, D. B. Lakings, and M. J. Ruwart. Int. J. Pharm. 731:231, 1991.

173. A. Adedoyin, P. R. Perry, and G. R. Wilkinson. Drug Metab. Dispos. 21:184, 1992.

174. A. Bertrams and K. Ziegler. Biochim. Biophys. Acta 1073:213, 1991.

175. A. Bertrams and K. Ziegler. Biochim. Biophys. Acta 1091:337, 1991.

176. K. Sathirakul, H. Suzuki, T. Yamada, M. Hanano, and Y. Sugiyama. J. Pharmacol. Exp. Ther. 268:65, 1993.

177. D. K. F. Meijer, W. E. M. Mol, M. Müller, and G. Kurz. J. Pharmacokinet. Biopharm. 18:35, 1990.

178. V. H. L. Lee. J. Control. Release 13:213, 1990.

179. P. Wüthrich, M. Martenet, and P. Buri. Pharm. Res. 11:278, 1994.

180. R. E. Stratford, Jr., and V. H. L. Lee. Int. J. Pharm. 30:73, 1986.

181. R. E. Stratford, Jr., and V. H. L. Lee. J. Pharm. Sci. 74:731, 1985.

182. A. Yamamoto, E. Hayakawa, and V. H. L. Lee. Life Sci. 47:2465, 1990.

183. I. K. Chun and Y. W. Chien. J. Pharm. Sci. 82:373, 1993.

184. L. Wang, D. Toledo-Velasquez, D. Schwegler-Berry, J. K. H. Ma, and Y. Rojanasakul. Pharm. Res. 10:1662, 1993.

185. P. P. Constantinides, J. P. Scalart, C. Lancaster, J. Marcello, G. Marks, H. Ellens, and P. L. Smith. Proc. Int. Symp. Control. Release Bioactive Mater. 20:184, 1993.

186. K. Morimoto, H. Yamahara, V. H. L. Lee, and K. J. Kim. Pharm. Res. 10:1668, 1994.

187. C. A. Barth, B. Lunding, M. Schmitz, and H. Hagemeister. J. Nutr. 2195:2200, 1993.

188. P. Langguth, H. P. Merkle, and G. L. Amidon. Pharm. Res. 11:528, 1994.

189. M. A. Hussain, M. S. Lim, K. S. Raghavan, N. J. Rogers, R. Hidalgo, and C. A. Kettner. Pharm. Res. 9:626, 1992.

190. M. A. Hussain, A. B. Shenvi, S. M. Rowe, and E. Shefter. Pharm. Res. 6:186, 1989.

191. M. A. Hussain, C. A. Koval, A. B. Shenvi, and B. J. Aungst. J. Pharm. Sci. 79:398, 1990.

192. T. F. Zioncheck, S. A. Chen, L. Richardson, M. Mora-Worms, C. Lucas, D. Lewis, J. D. Green, and J. Mordenti. Pharm. Res. 11:213, 1994.

193. R. Machida, T. Tokumura, Y. Tsuchiya, A. Sasaki, and K. Abe. Biol. Pharm. Bull. 16:43, 1993.

194. J. Okolicany, G. A. McEnroe, G. Y. Koh, J. A. Lewicki, and T. Maack. Am J. Physiol. 263:F546, 1992.

195. S. Yanai, Y. Sugiyama, T. Iga, T. Fuwa, and M. Hanano. Pharm. Res. 8:557, 1991.

196. H. Sato, T. Terasaki, K. Okumura, and A. Tsuji. Pharm. Res. 8:563, 1991.

197. T. Bui, C. Faltynek, and R. J. Y. Ho. Pharm. Res. 11:633, 1994.

198. R. Mehvar and T. L. Shepard. J. Pharm. Sci. 81:908, 1992.

199. A. L. Gerbes, R. Witthaut, V. Gulberg, G. Thibault, M. Bilzer, and D. Jungst. Hepatology 16:790, 1992.

200. S. H. Jang, S. H. Lee, S. H. Ryoo, S. H. Kim, and M. G. Lee. Int. J. Pharm. 84:273, 1992.

201. D. C. Kim, M. Hanano, Y. Kanai, N. Ohnuma, and Y. Sugiyama. Pharm. Res. 9:1394, 1992.

202. K. Mihara, T. Hojo, M. Fujikawa, Y. Takakura, H. Sezaki, and M. Hashida. Pharm. Res. 10:823, 1993.

203. K. Mihara, M. Mori, T. Hojo, Y. Takakura, H. Sezaki, and M. Hashida. Biol. Pharm. Bull. 16:158, 1993.

204. X. J. Zhou and N. D. Vaziri. Am. J. Physiol. 263:F812, 1992.

205. A. E. Mast, G. Salvesen, H. P. Schnebli, and S. V. Pizzo. J. Lab. Clin. Med. 116:58, 1990.

206. C. O. Beauchamp, S. L. Gonias, D. P. Menapace, and S. V. Pizzo. Anal. Biochem. 131:25, 1983.

207. T. Ogino, M. Inoue, Y. Ando, M. Awai, H. Maeda, and Y. Morino. Int. J. Pept. Protein Res. 32:153, 1988.

208. G. Stehle, H. Sinn, E. A. Friedrich, A. Wunder, H. H. Schrenk, J. Harenberg,P. Peschke, C. E. Dempfle, W. Maier-Borst, and D. L. Heene. J. Lab. Clin. Med. 122:728, 1993.

209. M. Nishikawa, Y. Ohtsubo, J. Ohno, T. Fujita, Y. Koyama, F. Yamashita, M. Hashida, and H. Sezaki. Int. J. Pharm. 85:75, 1992.

210. R. Langer and J. Folkman. Nature 263:797, 1976.

211. A. Hussain, J. Faraj, Y. Aramaki, and J. E. Truelove. Biochem. Biophys. Res. Commun. 133:923, 1985.

212. W. D. Rhine, D. S. T. Hsieh, and R. Langer. J. Pharm. Sci. 69:265, 1980.

213. M. V. Sefton, L. R. Brown, and R. S. Langer. J. Pharm. Sci. 73:1859, 1984.

214. R. Bawa, R. A. Siegel, B. Marasca, M. Karel, and R. Langer. J. Control. Release 1:259, 1985.

215. D. S. T. Hsieh, W. D. Rhine, and R. Langer. J. Pharm. Sci. 72:17, 1983.

216. L. Brown, L. Siemer, C. Munoz, and R. Langer. Diabetes 35:684, 1986.

217. T. T. P. Hsu and R. Langer. J. Biomed. Mater. Res. 19:445, 1985.

218. L. M. Sanders, J. S. Kent, G. I. McRae, B. H. Vickery, T. R. Tice, and D. H. Lewis. J. Pharm. Sci. 73:1294, 1984.

219. L. M. Sanders, B. A. Kell, G. I. McRae, and G. W. Whitehead. J. Pharm. Sci. 75:356, 1986.

220. H. M. Patel and B. E. Ryman. FEBS Lett. 62:60, 1976.

221. A. L. Weiner, S. S. Carpenter-Green, E. C. Soehngen, R. P. Lenk, and M. C. Popescu. J. Pharm. Sci. 74:922, 1985.

222. D. A. Eppstein, Y. V. Marsh, M. Van der Pas, P. L. Felgner, and A. B. Schreiber. Proc. Natl. Acad. Sci. USA 82:3688, 1985.

223. W. C. Koff, S. D. Showalter, B. Hampar, and I. J. Fidler. Science 228:495, 1985.

224. D. Gurari-Rotman and P. I. Lelkes. Biochem. Biophys. Res. Commun. 107:136, 1982.

225. B. C. Amundson and J. E. Wheaton. Biol. Reprod. 20:633, 1979.

226. J. F. Obie and C. W. Cooper. J. Pharmacol. Exp. Ther. 209:422, 1979.

227. J. W. Fara. Methods Enzymol. 112:470, 1985.

228. T. Heya, Y. Mikura, A. Nagai, Y. Miura, T. Futo, Y. Tomida, H. Shimizu, and H. Toguchi. J. Pharm. Sci. 83:798, 1994.

229. R. Gref, Y. Minamitake, M.T. Peracchia, V. Trubetskoy, V. Torchilin, and R. Langer. Science 263:1600, 1994.

230. T. Uchida, S. Martin, T. P. Foster, R. C. Wardley, and S. Grimm. Pharm. Res. 11:1009, 1994.

231. P. T. Golumbek, R. Azhari, E. M. Jaffee, H. I. Levitsky, A. Lazenby, K. Leong, and D. M. Pardoll. Cancer Res. 53:5841, 1993.

232. G. W. Creasy and M. E. Jaffe. Adv. Drug. Deliv. Rev. 6:51, 1991.

233. I. C. A. F. Robinson. Adv. Drug Deliv. Rev. 6:57, 1991.

234. J. Kost and R. Langer. Adv. Drug Deliv. Rev. 6:19, 1991.

235. F. Fischel-Ghodsian, L. Brown, E. Mathiowitz, D. Brandenburg, and R. Langer. Proc. Natl. Acad. Sci. USA 85:2403, 1988.

236. S. Sato, S. Y. Jeong, J. C. McRea, and S. W. Kim. J. Control. Release 1:67, 1984.

237. J. Heller, A. C. Chang, G. Rodd, and G. M. Grodsky. J. Control. Release 13:295, 1990.

5
Membrane Transport

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I. Introduction

A major problem in the development of pharmaceutical therapies is the delivery of the drug to the desired site of action. This may be illustrated by considering

the problems encountered in devising therapies for Parkinson's disease. The disorder involves a disturbance of monoamine metabolism in the basal ganglia in the brain, and a most successful therapy to alleviate the incapacitating symptoms is oral doses of levodopa (L-dopa). The drug is absorbed into the blood-stream in the gastrointestinal tract, passes across the blood-brain barrier, and is then taken up into neurons, where it is converted to dopamine. Oral doses of dopamine are ineffective because dopamine is unable to permeate the blood-brain barrier. The selective permeability of the blood-brain barrier to L-dopa vs. dopamine is a key factor in the success of this therapy. In fact, the delivery of any drug to any target cell in the body requires an appreciation of the permeability properties of membranes separating body compartments. The first problem to be addressed in drug delivery is the route of administration, generally oral or transdermal, the second is the distribution of the drug between the fluid compartments of the body, the third is the uptake into cells, and the final problem is the excretion of the drug via the liver and kidneys. In this chapter I will review the transport properties of plasma membranes and epithelial cells, and briefly consider some of the strategies to deliver drugs across membranes.

II. Plasma Membranes.

A simple three-dimensional representation of a plasma membrane is shown in Figure 1. The primary structure is a bimolecular film of lipid 5 nm thick that separates the intracellular and extracellular fluid compartments. The lipids are mainly glycerides (e.g., phospholipids: phosphatidylserine and phosphatidylinositol containing saturated and unsaturated fatty acids), and sterols (e.g., cholesterol). The bilayer of amphipathic lipids is thermodynamically stable, and it accounts for many of the properties of plasma membranes. For example, the lipid bilayer accounts for the electrical capacitance (0.51 mF cm^{-2}) and resistance (> 5,000 ohms cm^2), low permeability to polar ions and molecules, and the high permeability to hydrophobic molecules.

Two classes of proteins are associated with plasma membranes: intrinsic and extrinsic membrane proteins. Intrinsic proteins are globular proteins that form an integral part of the membrane and can only be removed from the lipid bilayer by detergents. These proteins generally span the bilayer and are held in place by hydrophobic interactions with the acyl chains in the hydrophobic core of the membrane, and by electrostatic interactions at both external surfaces of the membrane. These intrinsic proteins are responsible for the transport of polar ions and molecules across the membrane (i.e., these proteins form channels, carriers and pumps). Extrinsic membrane proteins, about 40% of the total membrane protein, are attached to the cytoplasmic face of the plasma membrane by weak electrostatic forces (Fig. 1). As a consequence, they can be removed by mild procedures

such as increasing the pH or ionic strength. These proteins,

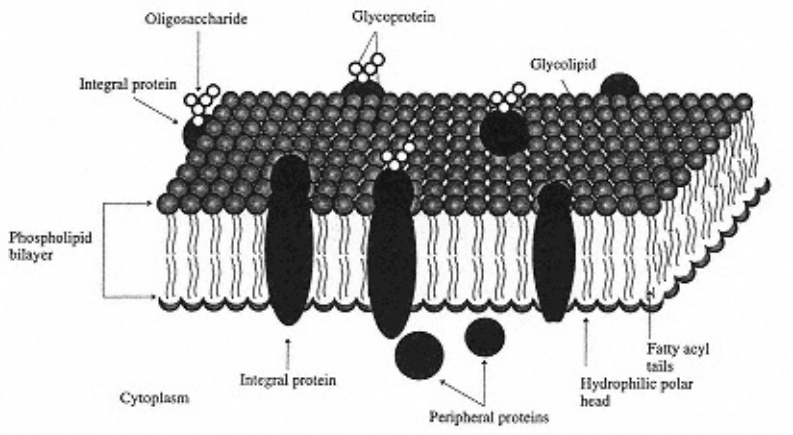

Figure 1
A structural model for plasma membranes. The membrane is composed of a bimolecular leaflet of phospholipid with the polar head groups facing the extracellular and cytosolic compartments and the acyl tails in middle of the bilayer. Sterols (e.g., cholesterol) are inserted between the acyl chains, but for simplicity are not shown. Integral membrane proteins are embedded in the lipid bilayer, and stability is ensured by hydrophobic bonding with membrane lipid and electrostatic bonding with polar head groups and the aqueous solution on each side of the membrane. The integral proteins are glycosylated on the exterior surface, and may be phosphorylated on the cytoplasmic surface. Extrinsic membrane proteins, peripheral proteins, (e.g., spectrin) are linked to the cytosolic surface of the intrinsic proteins by electrostatic interactions. (Ref. 1.)

even though they play an important role in anchoring membranes to the cell cytoskeleton, are beyond the scope of this discussion. Interested readers are referred to the introductory textbooks (e.g., [1]) for a general discussion of this topic.

III. Transport Mechanisms

There are six general mechanisms for the transport of substances across plasma membranes (Table 1), and each will be considered in turn.

A. Solubility/Diffusion

This term is used to describe the transport of substances across the lipid bilayer of the plasma membrane. The direction and rate of transport (J, moles s^{-1}) is determined by the concentration gradient of the substance across the membrane (C_o - C_i, moles l^{-1}, where the subscripts o and i refer to the inside and outside of the cell), the permeability of the bilayer to the particular substance (P, cm

Table 1 Membrane Transport
Mechanisms
Solubility/diffusion
Channels or pores
Facilitated diffusion
Active transport
Secondary active transport
Endocytosis

s^{-1}), and the area of the membrane (A, cm^2):

$$J = P\ A\ (C_o - C_i)\ (1)$$

In the case of human erythrocytes P values range from as low as 1×10^{-10} cm s^{-1} for erythritol to as high as 1×10^{-4} cm s^{-1} for propanol (see Table 2.1 [2]). A similar range of Ps is observed for plant cells, epithelia, model phospholipid membranes.

What accounts for this millionfold range in the permeability of membranes? A clue comes from the empirical observations made over the past 100 years that the permeability of cell membranes to nonelectrolytes is governed by their lipid solubility, or put more correctly, their partition coefficients (K) between lipid and water. Figure 2 show a plot of permeability against olive oil partition coefficients for an epithelial tissue. Note the linear relationship between P and K over a 10,000 fold range in P for all but the very small molecules.

The relationship between permeabilities and partition coefficients can readily be derived for the simple case when the rate-limiting step in permeation is the diffusion of the solute across the bilayer (Fig. 3).

Under these circumstances, the solute is at equilibrium between the aqueous solutions and the membrane $(\text{i.e., } C_1^m / C_1 = C_2^m / C_2 = K_m)$, where K_m is the partition coefficient for the solute between the aqueous solution and the membrane lipid. In the steady state the flux of the solutes in the membrane is given by Fick's law of diffusion:

$$J = -D_m\ A\ dC/dx = D_m\ A\ (C_1^m - C_2^m) / d \quad (2)$$

where D_m is the solute diffusion coefficient in the membrane lipid, and d is the thickness of the membrane. By using the partition coefficient equation (2) can be rearranged to express the flux in terms of the known solute concentrations in the external aqueous solutions,

$$J = D_m\ K_m\ A\ (C_1 - C_2)\ /d$$

or

$$J = P\ A\ (C_1 - C_2)$$

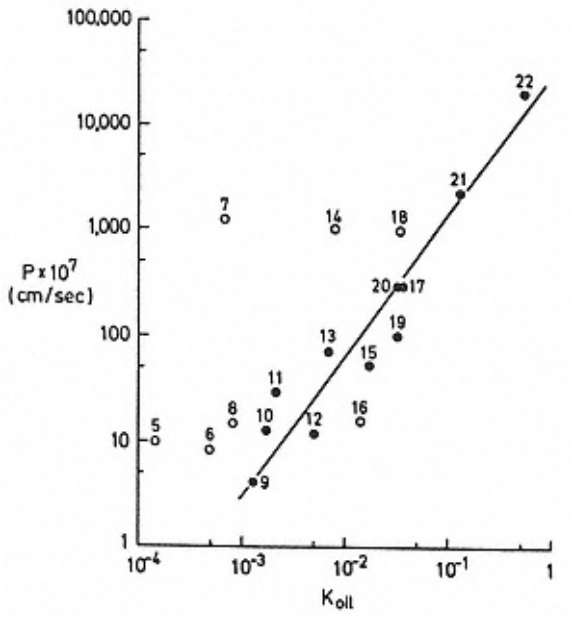

Figure 2
Experimental relationship between permeability (P) and partition coefficients (K) for the toad urinary bladder. Log P is plotted against log K_{oil}, and the line of identity is drawn to fit all but the smallest molecules (MV < 60 ml/mole). P is proportional to $K_{oil}^{1.3}$. Note that the small molecules (58 and 14) have higher Ps than expected from their K_{oil} values. Molecules: urea (5); ethylene glycol (6); water (7); acetamide (8); 1,3-propanediol (9); 1,2-propanediol (10); 1,4-butanediol (11); nicotinamide (12); 1,6-hexanediol (13); methanol (14); n-butyramide (15); isobutyramide (16); 1,7-heptanediol (17); ethanol (18); antipyrine (19); caffeine (20); n-propanol (21); and butanol (22). (From Ref. 29 with permission.)

where

$$P = D_m K_m / d \quad (3)$$

It follows from equation (3) that for solutes of approximately the same size (i.e. approximately the same diffusion coefficient) permeabilities are directly proportional to the solute partition coefficients. What governs the partition of solutes between water and lipid? At least with simple bulk systems (such as olive oil/H_2O and octanol/H_2O) both the molecular forces involved and the thermodynamics are well understood. The dominant factor in determining partition coefficients is the electrostatic interaction of the solute with water: the inter-

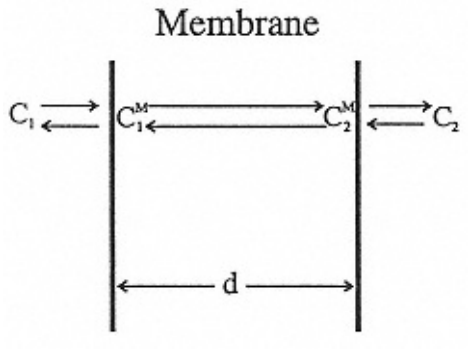

Figure 3
A simple scheme for permeation through a lipid bilayer by solubility/diffusion. The molecules partition between the aqueous phases and the membrane lipid according to their concentrations and partition coefficients, and diffuse within the membrane from one surface to another according to Fick's law.

molecular forces between solutes and water are generally considerably stronger than those between solute and lipid. Ions and ionized molecules have low partition coefficients and permeabilities due to strong ion-dipole and dipole-dipole interactions with water, while polar neutral molecules hydrogen bond with water. For example, the addition of one hydroxyl group to a simple hydrocarbon may reduce partition coefficients and permeabilities over 100-fold.

To a first approximation, there is a quantitative inverse relationship between log permeability and the number and strength of H-bonds that simple molecules can make with water. Group theory (see [3]) provides a satisfactory foundation for these experimental observations. Chemists are more familiar with the analysis based on Hammett sigma constants and Taft steric parameters, and the more recent FRAGMENT methods for calculating partition coefficients (see [4]). These also take into account intramolecular H-bonding and inductive effects between substitute groups on more complex molecules. The work by Hansch and Leo [4] also includes a comprehensive listing of partition coefficients (> 14,500) for model systems.

While the H-bonding ability of solutes plays a dominant role in determining partition coefficients, it is also necessary to include hydrophobic bonding to account for the increase in partition coefficients with increasing hydrocarbon content. Each additional methylene group on a molecule increases partition and permeability coefficients by up to 10-fold. As may be anticipated, an increase in hydrophobic bonding, increased van der Waals interactions between the molecule and the membrane hydrocarbons, partially accounts for this phenomenon,

but a major factor is the increase in entropy of the aqueous phase that pushes hydrocarbons out of water into the lipid environment (see [3]).

Despite the fact that there is a very good correlation between premeability coefficients and bulk phase partition coefficients over many orders of magnitude, (see Fig. 2), it is difficult to imagine that a bulk solvent is an appropriate model for a phospholipid membrane. The hydrocarbon of the membrane is highly ordered relative to a bulk solvent, and this must influence both the partition and diffusional processes. This is reflected practically in the size dependence of permeation. Figure 4 shows the size dependence of diffusion across an epithelial membrane. Diffusion constants were estimated by dividing the permeability coefficients (Fig. 2) by the estimated partition coefficients for this membrane

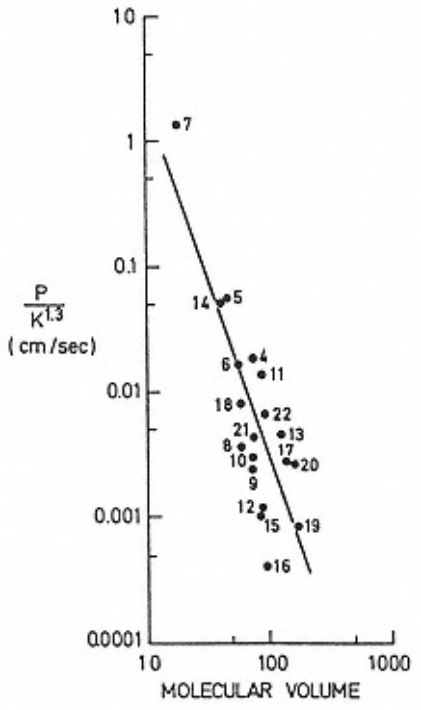

Figure 4
Membrane diffusion coefficients as a function of molecular volume. Membrane diffusion coefficients were estimated from the relation $D_m = P/K$, where K the membrane partition coefficient is approximated by $K_{oct}^{1.3}$ (Fig. 2). Log P/K1.3 is plotted against molecular volume. All numbered points refer to the compounds in Figure 2. (From Ref. 29 with permission.)

($K_{oil}^{1/3}$). The plot of Dm against molecular volume shows that membrane diffusion is proportional to $MV^{2.7}$, considerably higher than for diffusion in bulk solution (D proportional to $MV^{0.33}$). While this size dependence may be largely due to the presence of channels and/or carriers in this biological membranes (see below), a steep size dependence has also been obtained for pure lipid membranes (see [2]). This is reminiscent of diffusion in polymers, where diffusion depends on the motion of polymer molecules, the free space within the polymer, and the size and shape of the solute (see [5]). Even the partition of solutes into polymer membranes depends on the availability of holes or defects, and this would account for the high enthalpy of partition and positive entropies of solution of solutes in membranes.

These solubility/diffusion considerations apply to the permeation of drugs across the lipid bilayer component of all plasma membranes in the body, irrespective of the tissue or cell. However, the absolute rate of permeation may vary according to the precise lipid composition and physical state, frozen or fluid, of the particular membrane. Thus one may increase or decrease the rate of drug transport across all membranes in the body simply by changing nonessential substituent groups on the compound, and the magnitude of the effect can be estimated using group theory or the fragmentation method [3,4].

IV. Membrane Transport Proteins

Solubility/diffusion through the lipid bilayer may be sufficient to explain the rate and selectivity of nonpolar solute transport across plasma membranes. However, to account for the transport of ions and very polar solutes one has to turn to the role of the integral membrane proteins. In the 1950s and 1960s considerable success was recorded in cataloguing and characterizing specific membrane transport systems in every cell of the body. These were operationally defined as channels, carriers, and pumps, and, in some cases, where the abundance of the transport protein was very high, the proteins responsible were identified (e.g. the renal Na/K-pump and the red cell Cl/HCO_3 exchanger). While these transport proteins could account for up to 25% of the total membrane protein, the majority of transport proteins are present in low abundance (< 0.1% of the total protein). The low abundance, greasy nature, and lability of transporters were major obstacles to the identification and isolation of the proteins. These problems were overcome by the remarkable advances in molecular biology that led directly or indirectly to the cloning of transport proteins. Success initially depended on obtaining microsequence information, or antibodies, to screen cDNA libraries, and this resulted in the cloning of several ion pumps and channels in the mid-1980s. Shortly afterward advances in expression cloning eliminated the need for either protein purification or antibodies. In 1987 my laboratory was the first to clone a transporter using this method

[6]. By mid-1994, dozens of membrane

channels, carriers and pumps have been cloned, sequenced, and studied in heterologous expression systems.

Our understanding of the structure of transport proteins has not kept pace with the cloning of these proteins, and this has to do with the difficulty of obtaining three-dimensional crystals of membrane proteins. A three-dimensional crystal structure at the atomic resolution has only in the case of the photosynthetic reaction center of *Rhodopseudomonas viridis,* but lower-resolution structures have been obtained from highly ordered two-dimensional arrays of bacteriorhodopsin and an acetylocholine-gated cation channel in membrane preparations. On the basis of such images, other structural information, and theoretical considerations, it is now postulated that membrane spanning domain of the transport proteins is composed of bundles of 612 alpha-helices. The number of helices making up the bundle varies from one class of transport proteins to another; for example, the voltage-gated ion channels have 6 and most carriers have 12. Each helix lies perpendicular to the membrane and is composed of about 21 nonpolar amino acids, and one is linked to the next by chains of hydrophilic residues (see Fig. 9). Most charged and polar residues are located in the hydrophilic domains of the protein, but those found in the membrane-spanning helices must be important in stabilizing the structure by electrostatic bonding to other polar residues within the membrane domain. Glycosylation and phosphorylation only occur at the external and internal hydrophilic domains of the protein, respectively. At present much attention is being given to both theoretical and experimental approaches to determining the topology of cloned membrane transport proteins. The remainder of this chapter will be devoted to a brief summary of our present knowledge about plasma membrane channels, carriers, and pumps.

A. Channels

The major evidence for the existence of small aqueous pores or channels in biological membranes has been that the permeability of small polar molecules was much higher than expected from their partition coefficients. This is illustrated in Figure 2 by the fact that the permeability for water is 1000 times higher than predicted, and the permeability for urea is 10100 times higher than predicted. The permeabilities of slightly larger polar molecules such as mannitol and L-glucose are as predicted from their low partition coefficients. This led to the concept of membrane pores or channels, in which small polar molecules pass through aqueous channels (radii of around 4Å) and not through the lipid. Acceptance of this was not universal, especially in view of experiments showing anomalous high permeation of small polar solutes through artificial phospholipid membranes.

1. Ion Channels

Convincing experimental evidence for channels in biological membranes was first gathered for ions in the 1970s. Perhaps the most definitive experiment was that by Neher and Sakmann [7], who recorded single-channel currents from muscle fibers using a patch-clamp technique. Figure 5 shows the characteristics of channel currents. In this cell the currents flowing across a tiny patch of plasma membrane were recorded using a patch-pipette (A). At the applied membrane potential, -60mV, two channels open and close independently to pass currents of 2.4pA. Note that when the channels are closed no current flows across the membrane, but as each channel opens at irregular intervals for variable times

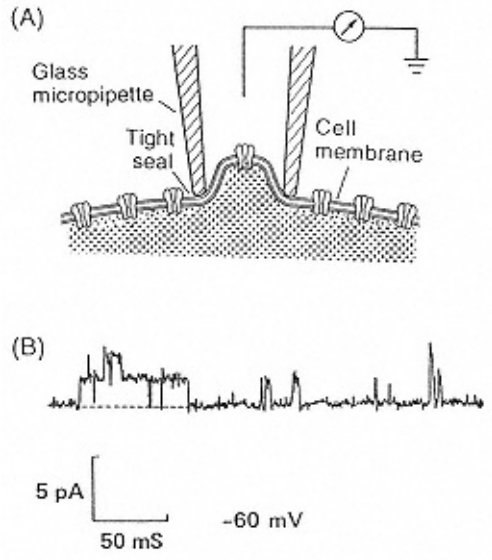

Figure 5
Recording current flow across ion channels. A. The patch-clamp technique used to isolate, voltage clamp, and record currents from a small patch of cell plasma membrane. A glass micropipette containing salt solution (tip diameter 1mm) is pressed against the cell membrane to form a tight seal, and the schematic electrical circuit is used to clamp the membrane potential at a fixed value and record the membrane currents. B. Channel currents were recorded from a patch containing two maxi-K channels on the apical membrane of frog choroid plexus epithelium. Currents were recorded over a time course of 250 ms when the pipette potential was held at -60mV. Note that current only flows across the membrane in short pulses, when the channel is open, and identical currents were

obtained at each opening. (Modified from Ref. 30.)

identical amounts of current (2.4pA) flow. Many types of channels for Na, K, Ca, and Cl have been since identified, and each has a diagnostic conductance (0.5-300pS), ion selectivity, and probability of opening (see [8]). Ion channel opening and closing may be controlled by either voltage or ligands, so-called voltage-gated and ligand-gated channels.

The nicotinic acetylcholine receptor channel is one of the best understood biological channels. The channel was first purified from the electric organ of the electric ray *Torpedo,* and shown to consist of four subunits arranged as a pentameric glycoprotein (2 alpha, 1 beta, 1 gamma, and 1 delta). Each of the four subunits has been cloned and sequenced, and functionally expressed in heterologous expression systems such as *Xenopus laevis* oocytes. The arrangement of the pentameric channel protein in the membrane was deduced from crystallographic analysis of electron microscope images of two-dimensional ordered arrays of *Torpedo* receptors in membranes [9]. Viewed face-on (Fig. 6), the central ion channel appears as a central well with a diameter of 2.5 nm surrounded by the five subunits. In the axial plane the channel appears to be occluded in the membrane domain, but the expected diameter of the channel controlling ion permeation (0.60.7 nm) is below the resolution of the projected image.

Ion channels belong to gene families with related properties and structures. One example is the voltage-gated channels for Na, K, and Ca. Each is characterized by a structural motif, six transmembrane alpha-helices with a high per-

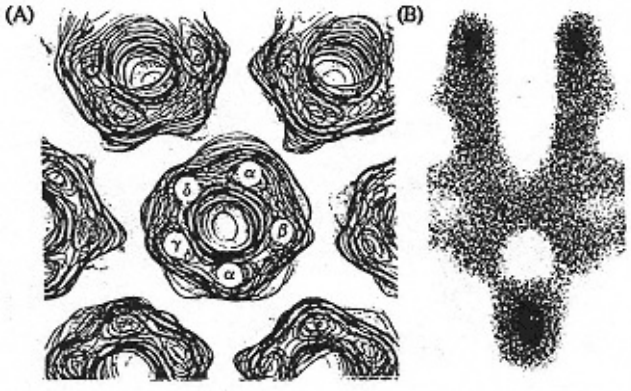

Figure 6
Image analysis of Torpedo acetylcholine gated ion channels. Electron micrographs were taken of unfixed, frozen arrays of channels in lipid membranes. A. Contour maps drawn to show the surface view; the probable arrangement of the subunits is given for one channel. B. An axial section of one channel shows how the protein is inserted into the lipid bilayer. (From Ref. 9 with permission.)

centage of identical and similar residues (Fig. 7). The functional channel is composed of four such motifs thought to be arranged around a central ion channel. The genes for the Na and Ca channels code for a single proteins containing all four motifs, while the gene for the K channel only codes for a single motif. This means that four K channel subunits are assembled into a functional channel at the posttranslational level. Despite the structural and functional homology between the channels, they are selective with their own unique pharmacology: Na channels are 12 times more selective for Na than other cations and are blocked by tetrodotoxin (TTX), while Ca channels are 1000 times more selective for Ca than other cations and are not blocked by TTX.

2. Water Channels

Definitive evidence for the presence of water channels came recently with the cloning of several proteins that increase water permeability when expressed in heterologous systems. The first, CHIP28 (e.g. [10]), is expressed in red cells

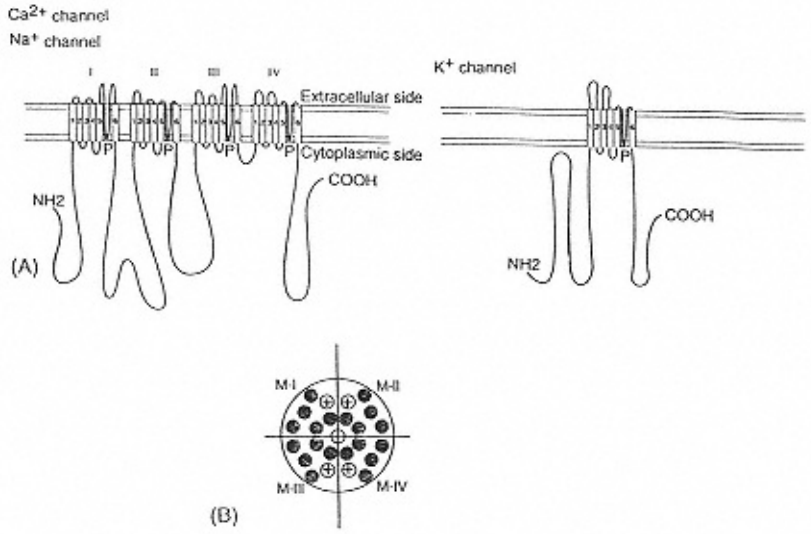

Figure 7
The secondary structure of voltage-gated ion channels. A. The Na, Ca, and K channels have a common structural motif, six transmembrane helices, a membrane-associated loop between helices 5 and 6, and two long N- and C- hydrophilic domains. Four of these motifs are thought to form a central ion channel B. Both the Na and Ca channel genes have four repeats that code for the entire channel, while the K channel gene only codes for one subunit. (After Ref. 31.)

and renal proximal tubule. It is a 28 kDa protein with six putative transmembrane alpha-helices, and when expressed in *Xenopus* oocytes it increased osmotic water permeability about 10-fold. The experimental results strongly suggest that CHIP28 is a water channel: the activation energy for water permeation is < 3 kcal/mol, whereas that for the native oocyte membrane is > 10 kcal/mole; water permeability is blocked by Hg while that in the native oocyte membrane is not; and CHIP28 does not increase ion permeability of the oocyte membrane. The selectivity of water channel for small polar solutes such as urea has not been reported, but the recent cloning of a separate urea transporter from kidney would argue for discrete water and urea permeation pathways.

B. Facilitated Diffusion

The simplest type of carrier-mediated transport is facilitated diffusion. The diagnostic features are summarized in Table 2, and a simple model is shown in

Table 2 Facilitated Diffusion

Permeability greater than predicted from solute's partition coefficient.
Transport is saturable.
Transport is stereospecific.
Isomers may be transported with different affinities.
Competition occurs between isomers.
Transport occurs down the solute's concentration gradient.

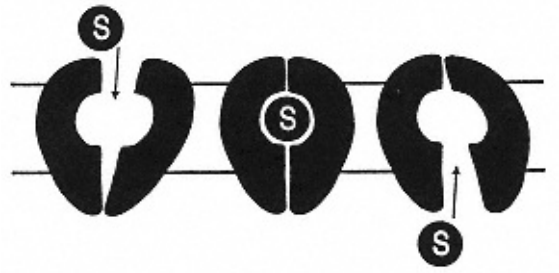

Figure 8
A simple model for facilitated diffusion of polar solutes across plasma membranes. Diffusion of a substrate S is mediated by a membrane protein that can exist in three different conformations: one in which S can bind from the outside of the cell; a second in which S is occluded; and a third in which the bound substrate is released to the cytoplasm. Transport can occur in either direction, and the direction of net transport depends simply on the substrate concentration gradient across the membrane.

Figure 8. Carriers are needed to transport polar substrates such as sugars and amino acids into cells. An important clue about their existence in cell membranes is that the rate of permeation of small polar solutes (e.g., urea [Fig. 2]), is much greater than expected from their partition coefficients and the general relationship between P and K_{oil}. Other differences between permeation via carriers and membrane lipid are that carrier-mediated transport is specific and saturable. As shown in the simple model (Fig. 8), carrier transport occurs via an integral membrane protein that has at least three conformational states: two where the ligand binding site faces either the external or internal surface of the cell, and a third where the bound ligand is occluded from both aqueous phases. Binding to the carrier at each membrane surface depends on the substrate concentration (S) and the affinity of the protein for the substrate (K_d). The direction of transport depends on the substrate contraction gradient, and the actual rate depends upon the density of the carrier proteins in the membrane, the probability that the binding site is occupied by substrate, and the rates of interconversion between the three carrier conformations.

While it is straightforward to derive the equations for this simple model, it has been quite difficult to determine experimentally the density of transporters, the K_ds for ligand binding at the two membrane surfaces, and the rate constants for the conformational changes: a minimum of 8 rate constants is required. It is customary to employ simple phenomenological equations to describe carrier transport:

$$J = J = \frac{[S]\, J_{max}}{K_m + [S]} \quad (4)$$

where J_{max} is the maximum rate of transport and K_m is the apparent affinity of the carrier for substrate. While J_{max} is proportional to the carrier density and K_m is related to K_d, both are a complex function of all the rate constants in the kinetic model. A more detailed discussion is beyond the scope of the present chapter.

The diagnostic features of facilitated diffusion (Table 2) follow directly from the model (Fig. 8) and equation (4). First, transport is saturable. This follows from the limited density of the carrier in the membrane and the turnover number. Turnover is determined by the rate constants for the interconversion of the conformational states, and values as high as 1×10^4 s^{-1} have been reported for the red cell glucose transporter. Second, transport is specific. This is determined by the structure of the active site for ligand binding, and as with enzymes the site can differentiate between isomers. Only D-glucose and not L-glucose is handled by the glucose transporter, but other D-isomers such as galactose and 2-deoxy-D-glucose are transported, albeit with a lower affinity (higher K_m). Third, competition occurs between isomers. This follows from the limited number of carriers in the membrane and the fact that isomers can be transported with different

Kms. Fourth, transport is downhill. The only energy available to drive the system is the substrate concentration gradient across the membrane. Hence the designation of this type of transport as facilitated diffusion.

The best-known carrier is the facilitated glucose transporter in human red cells [11]. Since the cloning of this transporter in 1985 it has become clear that there are at least six different human sugar transporters (GLUT1, GLUT2, GLUT3, GLUT4, GLUT5, and GLUT7) with different tissue distributions, kinetics, and substrate specificities. GLUT1 is the red cell D-glucose transporter and this is found also in the blood-brain barrier and placenta, GLUT4 is the insulin-regulatable D-glucose transporter in fat and muscle, and GLUT5 is the D-fructose transporter in the small intestine.

The sugar transport family now includes more than 100 transporters found in bacteria, plants and animals [12]. Most are evolutionarily related and have the structural motif of 12 transmembrane helices (Fig. 9). The family members carry out diverse transport functions ranging from facilitated sugar transport to H-driven sugar cotransport (see below), to the transport of nonsugar substrates (tetracycline, citrate, and quinate).

In mammalian cells there a number of facilitated transporters for amino acids (Table 3). The most well known is system L which handles neutral amino acids

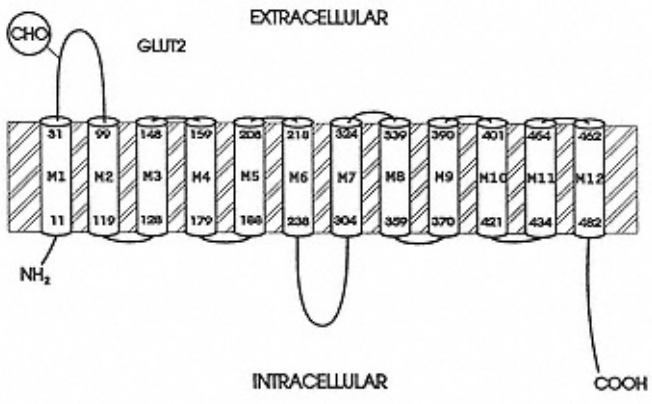

Figure 9
A model for the secondary structure of a facilitated glucose transporter (GLUT2). The 524 residue protein is predicted to contain 12 transmembrane helical domains with the N- and C- hydrophilic terminal regions facing the cytoplasm [19]. All hydrophilic loops connecting the membrane helical structures are short except those between M1-2 and M6-7. The M1-2 loop has one N-linked glycosylation site. More than 100 members of the GLUT gene family have this secondary structure profile, and there is some evidence for gene duplication between the N-terminal and C-terminal halves of the protein.

Table 3 Amino Acid Transport Systems

System	Ion	Typical substrates
Facilitative		
y+		Arginine, lysine
bo+		Lysine, cystine
L		Phenylalanine, leucine
Concentrative		
B	Na	Threonine, alanine
ASC	Na	Alanine, glycine
IMINO	Na	Proline, pipecolic acid
A	Na	MeAIB
b	Na and Cl	b-Alanine
X^{+}_{AG}	Na and K	Glutamate, aspartate

such as phenylalanine and leucine with high affinity. Cationic and neutral amino acids are transported by systems b_{O+} and y+. When the cDNA coding for the murine ecotropic retrovirus receptor is expressed in *Xenopus* oocytes it transports amino acids handled by system y+. This suggests that this protein has a dual function as a transporter and receptor (see [13]). In the case of charged amino acids, the rate of facilitated diffusion is determined by the concentration gradient and the membrane potential. A number of laboratories have also isolated a small family of clones (NAA, rBAT, D2) that stimulate the uptake of neutral and an cationic amino acids when expressed in oocytes, and the specificity and kinetics of uptakes resemble the b_{O+} system. It is unclear yet whether these cDNAs code for the transporter or a regulator of endogenous oocyte transport. These clones facilitate the uptake of cystine, and it is notable that cystinuria, a defect in the renal absorption of cystine, has been linked to missense mutations in the rBAT gene [14].

C. Pumps

The pumps are carrier proteins that are able to transport ions against their electrochemical potential gradients using ATP as an energy source. In animal cells the Na/K-pumps, also known as Na/K-ATPases, are responsible for maintaining the intracellular Na and K concentrations, and in epithelial cells of the intestine and kidney are responsible for the absorption of salt and water. The Na and K gradients across plasma membranes set the membrane potential, enable the generation of action potentials in excitable cells, and drive the active transport of sugars, amino acids, neurotransmitters, osmolytes, xenobiotics and other ions (PO_4, I, Cl, Ca etc) into cells. So important are Na/K-pumps in the human body

that more than 20% of the oxygen consumed by the body at rest is used in the synthesis of ATP utilized by these pumps.

The Na/K-pump transports 3 Na ions out of the cell and 2 K ions into the cell at the expense of one molecule of ATP (Fig. 10). The obligatory coupling of Na and K transport means that the pump rate depends on the intracellular $[Na]_3$ concentration and the extracellular $[K]_2$ concentration: the apparent affinity for Na is close to the normal intracellular Na concentration, 10 mM, and the apparent affinity for K is close to the normal extracellular fluid K concentration, 4 mM. The 3/2 coupling coefficient means that for each cycle of the pump there is net movement of cations out of the cell, and this compensates for the passive leak of Na into the cell down the steep electrochemical potential gradient (approx. -150mV), and enables the cell to maintain its volume.

As indicated in Figure 10 the Na/K-pump involves a phosphorylated intermediate, and the catalytic cycle has two major conformations: E_1 the conformation with ion-binding sites facing the cytoplasm; and E_2 the conformation with the ion-binding sites facing the exterior of the cell [15]. In addition, there are states where the ions bound to E_1 and E_2 are buried, occluded, within the protein. Cardiac glycosides are potent specific inhibitors of the Na/K-pump, and they appear to compete for the K binding to E_2P.

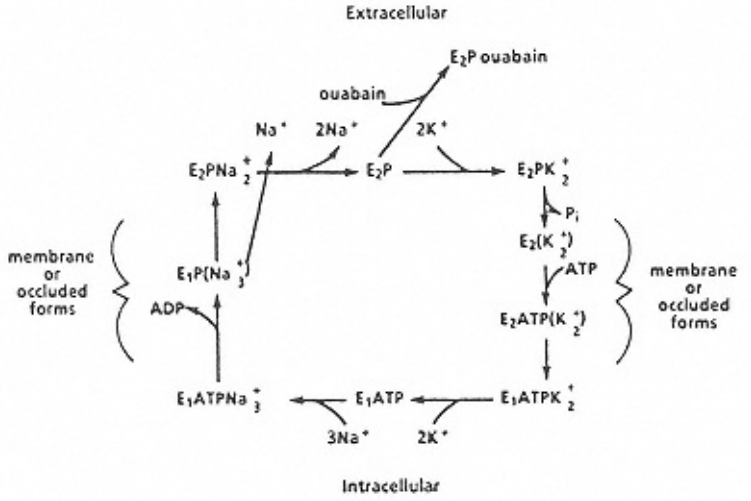

Figure 10
A reaction scheme for the 3Na/2K-pump (ATPase) shows the sequence of ion binding, the phosphorylation step, and the steps in which the ions are occluded from the external aqueous phases. (From Ref. 16.)

Na/K-pumps are composed of two subunits in equimolar proportions [16]: One is a 113kDa alpha subunit; and the other is a 35kDa glycosylated beta subunit. There are at least 3 isoforms of both the alpha and beta subunits, and these are expressed in different combinations in cells throughout the body (e.g. alpha3beta2 is found in neural tissues). The alpha subunit contains the ATP binding site, the phosphorylation site, and the residues essential for ion binding, while the beta subunit appears to be involved in the targeting and stabilization of the pump in the plasma membrane. Isoforms of the two subunits have been cloned and sequenced, and it is clear that the Na/K-pump belongs to a family of pumps found in plasma membranes. The family includes the gastric H/K-pump and the sarcoplasmic reticulum Ca-pump. The Na/K- and H/K-pumps share homologous alpha and beta subunits and a similar catalytic cycle, but the Ca-pump does not have a beta subunit. While there is some disagreement about the secondary structure of the alpha subunits, a consensus is growing that they contain 10 transmembrane helices. Considerable effort is underway to determine the topology of the pumps and to identify the residues and domains involved in ATP, ligand, and inhibitor binding (see [16]). The beta subunit of the Na/K- and H/K-pumps appears to have only one transmembrane helical domain, with most of the mass of the protein on the extracellular surface of the membrane. There is evidence that the beta subunit of one pump will substitute for the other in producing functional pumps.

D. Cotransporters and Exchangers

Many other ions and solutes are accumulated in cells against their electrochemical potential gradients. Examples include the accumulation of iodide by the thyroid gland, the accumulation of amino acids into liver cells, the reuptake of neurotransmitters into neurons, and the absorption of sugars and phospate by the small intestine. Before 1960 all these processes were assumed to be due to active transport. In 1960 Bob Crane proposed that the active transport of glucose across the brush border of the small intestine is driven by the Na gradient across the plasma membrane (i.e., glucose transport is coupled to Na transport and that uphill sugar transport is energized by the Na gradient). The process is called Na/glucose cotransport by physiologists and Na/glucose symport by biochemists. Cotransport and exchange are frequently referred to as secondary active transport. Over the next decade this hypothesis was tested, confirmed, and expanded to include the membrane potential as a driving force (i.e., the Na electrochemical potential gradient). Similar cotransport mechanisms were postulated to explain the active transport of other organic solutes in cells ranging from bacteria to plants to humans: H/lactose cotransport in *E. coli.,* H/sucrose cotransport in plant cells, and Na/amino acid cotransport in neurons and hepatocytes. It was even extended to include the transport of ions such as Cl, I, and PO4. In all these

cases Na or H gradients used to drive cotransport are set up and maintained by ion pumps.

1. Cotransporters.

Many of these cotransporters have been identified, cloned, sequenced, and expressed in heterologous expression systems such as *Xenopus* oocytes. Some belong to the glucose transporter family with a secondary structure profile very similar to that for the mammalian facilitated glucose cotransporters (Fig. 9), while others belong to different gene families. One family is the Na/glucose cotransporter (SGLT1) family where again the proteins consist of 12 transmembrane helices, but there is no homology to the GLUT family at the DNA, protein, or secondary structure levels (Fig. 11). The SGLT1 family members transport sugars, osmolytes, and amino acids, and are found in both bacteria and animal cells [17]. Other families include the H/peptide and Na/Cl/neurotransmitter cotransporters [18] (Table 4).

The most extensively studied cotransporter is the intestinal Na/glucose cotransporter (SGLT1). The SGLT1 gene on chromosome 22 codes for a 664 residue, 73kDa protein that is heavily glycosylated. There is some evidence that the protein functions as a homotetramer in the native membrane. The kinetics and specificity of the cloned protein have been examined in the *Xenopus* oocyte expression system [19]. The natural sugar substrates are glucose and galactose,

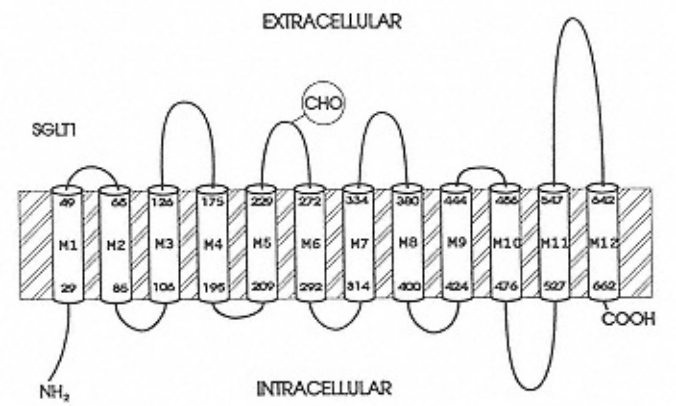

Figure 11
A secondary structure model for the Na/glucose cotransporter SGLT1 [19]. The 664 residue protein is predicted to have 12 transmembrane helices and both the N- and C- termini facing the cytoplasm. There is one N-linked glycosylation site on the hydrophilic loop between M5-6. As in the case of GLUT2 (Fig. 9), there is evidence for gene duplication between the N- and C-terminal halves of the protein. Note that the secondary structure profile is distinct from that for GLUT2.

Table 4 Cotransporter Families

Na/cotransporters (SGLT1 family)	
	Intestinal Na/glucose
Na/Cl/neurotransmitter and osmolyte cotransporters (GAT family)	
	Neurotransmitters: brain Na/Cl/GABA
	Osmolytes: renal Na/Cl/betaine
Na/K/glutamate	
	Brain Na/K/glutamate
Na/2Cl/K	
	Renal Na/2Cl/K
H/dipeptide	
	Intestinal H/glycyl-sarcosine
Na/bile salt	
	Liver Na/taurocholate
Na/phosphate	
	Renal Na/phosphate

but the transporter accepts a wide variety of analogs in the D-glucose conformation with a free hydroxyl group in the equatorial position at carbon 2. Na gradients drive sugar transport with a Na/sugar coupling coefficient of 2, but in the absence of Na we have found that proton gradients are quite effective in energizing transport. This implies that there are close functional similarities between the Na- and H-driven cotransporters. A model for the coupling of Na and sugar transport via SGLT1 is shown in Figure 12. According to this model there is ordered binding of Na and sugar to the transporter: Na binds first at the exterior surface and this produces a conformational change that allows sugar to bind. This is apparent as a decrease in sugar K_m on the addition of Na. Phlorizin, a competitive inhibitor of Na/glucose cotransport, acts by competing for sugar binding to the Na form of the transport protein. After the binding of ligands there is another conformational change of the protein that delivers the binding sites to the inferior face of the membrane, where both sugar and Na dissociate. The low intracellular Na concentration, 10 mM vs. 100 mM outside, ensures the dissociation of sugar even when the intracellular sugar concentration rises above the external level. The cycle is completed by the return of the ligand binding sites to the exterior face of the membrane. The rate-limiting step appears to be this final conformational change, and the turnover number is 525 s^{-1} orders of magnitude lower than that for the facilitated glucose cotransporters.

In humans a disorder of intestinal glucose and galactose absorption, glucose galactose malabsorption syndrome, is due to a defective Na/glucose cotransporter. In some patients

with the disease we have been able to identify the mutations responsible for the lack of sugar absorption. The mutations, causing

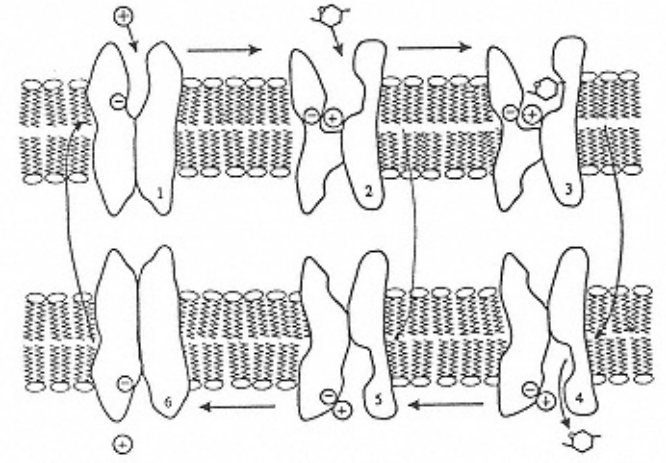

Figure 12
A model for Na/glucose cotransport [19]. This model will account for uphill transport of glucose from the extracellular fluid to the cytoplasm. The driving force for transport is the Na electrochemical potential gradient across the membrane: 150 mM Na out, 1020 mM Na in, and a membrane potential of -50mV. The membrane potential and the high external Na favor Na binding, state 2, and Na binding produces a conformation change that permits sugar to bind. The transporter then undergoes a conformational change to deliver Na and sugar to the internal surface, where the low intracellular Na concentration results in Na and sugar dissociation. The membrane potential then drives the final step of the transport cycle, which returns the ligand-binding sites to the external face of the membrane.

the substitution of Asp 28 with an Asn or Ala residue, block the transport of the protein from the endoplasmic reticulum (ER) to the plasma membrane.

The transport of amino acids is particularly complex: there are at least six different Na-dependent cotransporters with overlapping substrate specificities (see [13]). Some are solely Na-dependent, while others also require Cl and/or K. The complexity is exemplified by the glutamate transporters, which require Na and K for full activity. The one cloned from the intestinal mucosa, where it is presumably involved in glutamate absorption, is highly homologous with two from the brain that may be involved in synaptic transmission. This glutamate family is distinct from the Na/Cl neurotransmitter family, but is related to the brain Na-dependent neutral amino acid transporter that has characteristics similar to the widely expressed ASC system [20]. Table 3 lists the Na-dependent amino acid transporters that have been identified by kinetic, biochemical, or molecular techniques.

2. Antiporters

In addition to cotransporters in which the Na or H gradient across the membrane is used to drive the transport of an organic or inorganic substrate in the same direction as the ion flux, transporters exist in which the ion gradient is used to drive transport of a substrate in the opposite direction. These transporters are called antiporters or exchangers, and the best known examples are the Cl/HCO_3, Na/H, and Na/Ca exchangers. The red cell Cl/HCO_3 exchanger, which makes up 25% of the total membrane protein, was the first to be cloned [21], and now at least three members of this gene family are expressed in different tissues in the body. Two major domains in the exchanger have been correlated with the dual functions of the protein. A membrane-associated domain with 12 transmembrane helices is linked to anion exchange, while the hydrophilic N-terminus anchors the protein to the cytoskeleton. Recently the membrane domain was reconstituted into two-dimensional crystals in lipid; image analysis of negatively stained preparations showed that the protein is a dimer with a central depression that may be a channel for anion transport [22]. However, at a resolution of only 20 Å it is difficult to draw firm conclusions about structure/function of this exchanger.

A ubiquitous exchanger found in virtually all cells is the Na/H exchanger. It is involved in the control of cell volume, pH, growth, and Na transport. Four mammalian isoforms, NHE1, NEH2, NEH3 and NEH4, have been cloned and sequenced [23]. All are suggested to have two domains, an N-terminal region with 10 transmembrane helical domains and a hydrophobic region of 300 residues at the C-terminus, which is the target of the protein kinases. The membrane domain is the most highly conserved among the isomers, and at least one isomer, NHE1, appears to exist as a dimer in the membrane. Truncation experiments indicate that the membrane domain is involved in the transport of Na and H across the membrane, while the C-terminal cytoplasmic domain is required for regulation by growth factors, thrombin, and second messengers.

The kinetics of Na/H exchange indicate that there is one external Na binding site with a K_m of 16mM, while there are at least two independent internal H binding sites. One is a modifier site involved in the regulation of transporter activity. The diuretic amiloride and its analogs are specific and potent blockers of Na/H exchange, and two residues in the fourth transmembrane helix are thought to be involved in high-affinity binding to the exchanger. The diuretics appear to compete with Na for the external ion binding site on the protein. Apart from short-term regulation of transporter activity by phosphorylation of the hydrophilic cytoplasmic C-terminal region, there is strong evidence for long regulation of one isoform at the level of gene transcription by glucocorticoids.

An important third family of exchangers is that for Na/Ca exchange. In the heart this

exchanger plays a dominant role in the regulation of intracellular Ca, which in turn influences the force of contraction. The therapeutic effect of car-

diac glycosides on the heart is probably due to a decrease in Na/Ca exchange activity caused by a decease in the Na gradient across the membrane. The exchanger is a 120 kDa protein with 11 transmembrane helices, and a large hydrophilic loop between helices 5 and 6 has a calmodulin-binding site to regulate activity [24]. The transporter catalyzes the exchange of 3 Na ions for 1 Ca ion by a consecutive electrogenic mechanism. There is evidence that intracellular Ca also regulates the exchanger, and it is postulated that this is required to prevent depletion of intracellular Ca below a critical level.

E. Endocytosis

Phagocytosis, pinocytosis, and endocytosis are not usually considered in the discussion of membrane transport processes. Phagocytosis is the nonspecific uptake of extracellular material by a process in which the cell engulfs the particle to be ingested. The rate of uptake is directly proportional to the concentration in the extracellular fluid.

Pinocytosis is a similar nonspecific process, but in this case uptake is by invagination of the membrane to form an intracellular vesicle. The most specific process is receptor-mediated endocytosis, by which ligands bind to a specific membrane receptor before being internalized. Materials ingested into cells by these mechanisms are either stored in vesicles or degraded in lysosomes. The best known examples of receptor mediated endocytosis are those for immunoglobulins (the Fc receptor), transferrin, and low-density lipoproteins. Endocytosis occurs at specialized depressions of the plasma membrane called coated pits. Cell biology texts (e.g., [1]) should be consulted for further details.

V. Principles of Epithelial Transport

The chief function of epithelial cells is to provide barriers between the body and its environment and between body compartments. These barriers maintain the composition of the body and the differences in composition between body compartments. The simplest epithelia are those formed by a single layer of cubodial or columnar cells (e.g., the gallbladder). The cells are joined together by tight junctions (zonula occludens), adhering junctions (zonula adherens), desmosomes, and gap junctions. The presence and properties of the tight junctions determine the ability of ions and molecules to cross the epithelium between the cells, the extracellular pathway (Fig. 13). When present, the tight junctions provide a continuous seal around the entire circumference of the cell. The plasma membranes near the apical surface of cell actually fuse to occlude the space between adjacent cells. The gap junctions are involved in cell-cell coupling, while the desmosomes (belt desmosomes, spot desmosomes, and hemidesmosomes) contribute to the mechanical stability of the epithelium by holding the

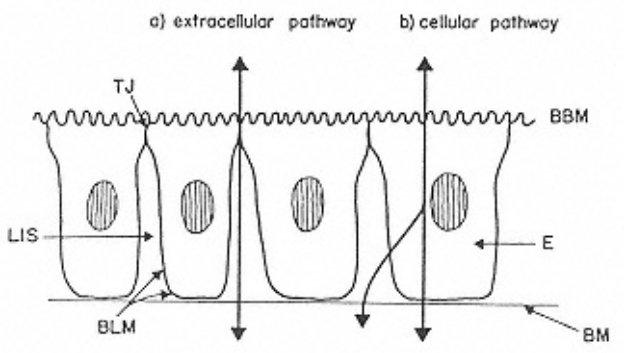

Figure 13
The two routes for permeation across epithelial cells: the extracellular and the cellular pathways. In epithelia (E) without tight junctions (TJ) there is free diffusion between the cells and the rate is determined by the fraction of the surface area occupied by the extracellular spaces and the concentration gradients across the epithelium. In tissues where tight junctions are present permeation is also controlled by the permeability properties of the junctions, and the cellular pathway. Transport across the cells is determined by two plasma membranes in series: the basolateral membrane (BLM) and apical or brush border membrane (BBM). In this case the structure of the lipid bilayer and the membrane transport proteins controls the rate and direction of transport. LIS = lateral intercellular space, BM = basement membrane.

cells together and to the basement membrane. In any given epithelium the barrier properties are determined by the properties of the cellular and extracellular (paracellular) pathways. The ependyma and pia covering the internal and external surface of the brain and most endothelia do not have tight junctions, and so ions, nutrients, and macromolecules permeate freely between cells. The electrical resistance of these membranes is very low (< 2 ohms cm2) and this is determined by the cross-sectional area and width of the intercellular spaces and the specific resistance of the interstitial fluid. Most epithelia have tight junctions, and so there is restricted diffusion between cells. The ion permeability of the tight junctions ranges from very leaky to very tight, and this is reflected in the electrical resistance of the epithelium. In tissues such as the choroid plexus, renal proximal tubule and gallbladder the junctions are very leaky to Na, K, and Cl, and the transepithelial resistance range from 5 to 25 ohms cm2. The unidirectional ion fluxes across the epithelia are very high and so these epithelia are unable to maintain ion gradients across the tissues. On the other hand, the urinary bladder, renal collecting tubule, and gastric mucosa maintain high ion gradients (e.g., the H gradient across the gastric mucosa can be as high as 1,000,000 / 1) and in these epithelia the electrical resistances can be as high as 40,000 ohms cm2. The structural basis of this huge range in

ion permeability of tight junctions is not clear.

When the permeability of the paracellular pathway is low, transport across the epithelium is determined by the cellular pathway (Fig. 13). In this case ions and molecules must pass across two plasma membranes in series, and the rate of transport depends on the permeability of the lipid bilayers and the presence of channels, carrier, pumps, and cotransporters. Thus the factors that control transport across the plasma membrane of single cells are the same factors that control transport across epithelia. Passive permeation of ions and molecules depends on the concentration gradients across the epithelium, the nature of the solute, and the presence of channels and carriers in the membrane. This is illustrated for simple non-electrolyte permeation across the urinary bladder in Figure 2.

A unique feature of epithelia is that the cells are polarized. The presence of tight junctions divides the cell into apical and basolateral surfaces, and the structure and composition of these two surfaces are generally quite different. In structural terms, the apical surface may be highly increased in area due to microvilli, whereas the basolateral membrane may be quite flat or extensively folded (e.g., the cells of the small intestine and renal proximal tubule). The composition of the plasma membranes is generally quite different in terms of lipid composition and transport proteins. The lipids of the cytoplasmic leaflet appear to be identical, but the lipids of the external leaflet are quite different at the two surfaces. Glycolipids are restricted to the external leaflet of the apical membrane, and, although they are free to diffuse in the lateral direction, they cannot pass the tight junction. Na/K-pumps are limited to the basolateral surface of enterocytes while Na/glucose cotransporters are only present in the brush border surface. When single cells are isolated from such a polarized epithelium (i.e., the junctions are disrupted) the membrane proteins redistribute around the entire surface of the cell.

A most fascinating question is how proteins and lipids are targeted to the apical and basolateral membranes of epithelia. In most epithelia the Na/K-pumps are only found in the basolateral membrane, but in a few (e.g., choroid plexus), the pumps are in the apical membrane. Likewise the Na/glucose cotransporter is found in the apical membrane of the enterocyte, but is in the basolateral membrane of the parotid gland. The factors determining the trafficking of membrane proteins from the ER to the plasma membranes of epithelia are being actively explored.

A. Design of Epithelial Transport Systems

Given the catalog of membrane proteins known to exist (see above), it is simple to design an epithelium to transport ions and molecules actively from one body compartment to another. For example, to design an epithelium to transport Na actively from the apical to the basolateral surface, simply placing Na channels

in the apical membrane and Na/K-pumps in the basolateral membrane would solve this problem. Furthermore, if the junctions are tight to Na, this arrangement of channels and pumps would enable the epithelium to generate and maintain a Na gradient between the two compartments. Na channels in the apical membrane would allow Na to enter the epithelium down the electrochemical potential gradient; the rate of entry would depend on the magnitude of the gradient, the number of channels, and the open time and conductance of the channels. Entry would cause the intracellular Na concentration to rise, but since the Na/K-pump rate depends on $[Na]^3$, a small rise in the intracellular Na would dramatically increase the pump rate. Depending on the density of the pumps, a new intracellular Na concentration would be reached at which the Na influx through the channel is balanced by the Na efflux through the pump. The net result is the transport of Na from the apical to basolateral surface of the cell. One consequence is that the intracellular K concentration would increase due to the increase in pump rate, and, depending on the distribution of K channels between the two surfaces of the cell, this will result in either a futile recycling of K across the basolateral surface or the net secretion of K across the epithelium. The apical Na channel can be replaced with either an Na/H exchanger or an Na-cotransporter in this model, and the rate of Na entry into the cell would then depend on the density and kinetics of the carrier in the membrane. Simply reversing the distribution of the channels (or carriers) and pumps in the cell would result in an epithelium that would actively secrete Na and absorb K.

One other example will suffice to illustrate the principle. Transport of nutrients across an epithelium against their concentration gradient can be accomplished by the judicious placement of facilitated carriers and contransporters on each side of the epithelium (Fig. 14). The absorption of glucose can be achieved by inserting a Na/glucose cotransporter in the apical membrane and a facilitated sugar transporter in the basolateral membrane. Glucose transport into the cell across the apical membrane is driven by the Na electrochemical potential gradient, and as the sugar concentration in the cell rises above the plasma level the basolateral transporter facilitates the exit of glucose out of the cell. The rate of net glucose contransport and the intracellular glucose concentration depends on the sugar concentration, and the density and kinetics of each transporter. This model requires Na/K-pumps to maintain the intracellular Na concentration, and if the pumps are on the basolateral surface it follows that glucose will stimulate the net absorption of Na. This in fact occurs in the small intestine, and is the basis of oral rehydration therapy for patients with cholera.

VI. Membrane Transport and Drug Delivery

As indicated in the Introduction, the delivery of drugs to targets within the body requires

an understanding of the structure and properties of membranes. Oral

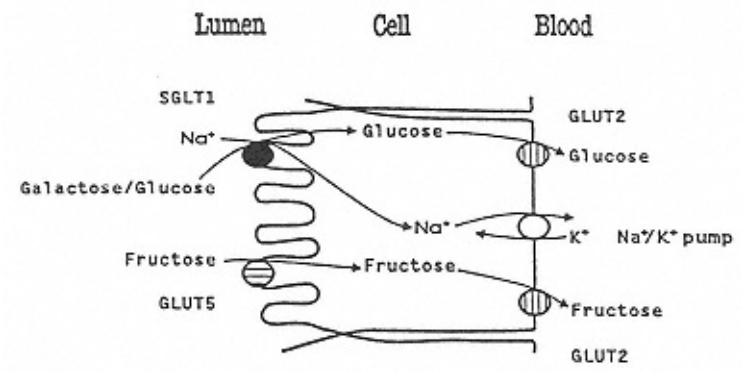

Figure 14
A model for sugar transport across the intestine. In this epithelium the enterocytes are attached by tight junctions near the brush border surface of cell. The junctions permit functional polarization of the cell with different transport proteins in the brush border and basolateral membranes. The Na/K-pumps and the facilitated sugar transporter (GLUT2) are restricted to the basolateral membrane, while the Na/glucose cotransporter (SGLT1) and facilitated fructose transporter (GLUT5) are restricted to the brush border membrane. Fructose transport across the epithelium is passive (i.e., down the concentration gradient) but is facilitated by the GLUT5 and GLUT2 transporters: GLUT5 is selective for fructose, whereas GLUT2 can transport fructose, glucose, and galactose. Glucose, and galactose, transport across the epithelium can occur against the sugar concentration gradients (i.e., the sugars can be transported from lumen to blood even when the sugar concentration in the lumen is lower than in blood). SGLT1 drives the uphill transport of sugar across the brush border membrane, and then glucose and galactose pass into the blood down their concentration gradients across the basolateral membrane via GLUT2. The Na that enters into the cell with sugar via SGLT1 is transported out of the cell by the basolateral Na/K-pump. Hence sugar in the gut lumen stimulates salt (and water) absorption. Similar polarized distributions of amino acid transporters also account for amino acid absorption across the intestine. (From Ref. 19).

delivery is usually the preferred route, but this requires absorption of the drug by the stomach and/or small intestine in a time-controlled manner. Only lipidsoluble drugs are likely to be efficiently absorbed by this route, but the pharmacologist has some flexibility in altering the rate of drug absorption by modifying the structure of the drug (i.e., by altering the partition coefficient with changes in substituent groups) (see above). Poor absorption and/or degradation of the drug in the gastrointestinal (GI) tract frequently requires that drugs be directly injected into the body intravenously (i.v.), intraperitoneally (i.p.), or intramuscularly (i.m.). Apart from considerations of issues such as drug/serum protein interactions, the transport of the drug from one body compartment to

another also requires an understanding of membrane transport. If the i.v. injected drug has to be delivered to cells in the brain, this involves transport from blood to brain across the endothelial cells of the brain microvasculature and the uptake into cells across the plasma membrane. Again this has usually been accomplished by increasing the concentration gradients (dose) and the lipid solubility of the drug. The obvious disadvantage is that this will also increase the delivery of the drug to all cells and tissues, with risks of undesirable side effects.

Can membrane transporters be used to overcome the problems of low permeability and nonspecificity? As discussed, membrane channels, carriers and pumps, and membrane transport systems are generally very specific and so it is difficult to design drugs that will be accepted by these systems. To date, most attention has been directed at the use of receptor-mediated endocytosis and transcytosis. The rationale is to complex drugs (e.g., peptides) to substrates for receptor-mediated endocytosis (see [25]). Potential targets are Fc receptor in the neonatal intestine, and the insulin and transferrin receptors in the blood-brain endothelium. The feasibility of the approach has been demonstrated in studies showing that a vasoactive intestinal peptide (VIP)-chimera was transported into the brain by the transferrin receptor to produce a pharmacological effect [26].

A different tactic that may prove useful is to exploit the specificity, or lack of specificity, of transporters for sugars, amino acids, osmolytes, and xenobiotics. The hope is that the substrate specificity is sufficiently broad to permit transport of the drug or prodrug. One example is the Na/glucose cotransporter (SGLT1) for which beta-glucosides are good transported substrates. Both phenyl and naphthyl glucosides are transported [27], and so it is possible that some prodrugs can be targeted to this transporter. Additional advantages of glycoside prodrugs are that their absorption from the GI tract may be enhanced by the presence of SGLT1 in the brush border of intestinal enterocytes and that the glycoside linkage may be readily cleaved by nonspecific glycosidases in cells to liberate the drug.

The second example is the transport of drugs by the H/oligopeptide cotransporters found in the small intestine and kidney [28]. Although the major role of these carriers is to transport dipeptides, it is clear that the cloned transporters also handle tripeptides, and peptide-derived antibiotics such as cephalexin and cyclacillin. It is to be expected that further specificity studies will lead to additional opportunities to employ transporters as drug delivery systems. In the longer term, as we determine how to regulate the expression of genes in cells and tissues, it may be possible to deliver a drug selectively to a target cell or tissue by controlling the expression of a transporter to accumulate the prodrug. Cotransporters are excellent target systems because of their unique tissue distribution in the body, their broad substrate specificity. Furthermore, the fact that Na and H gradients

drive the accumulation of substrates in cells to high

levels (> 10-fold higher than plasma levels) would permit use of much lower drug doses and so minimize side effects.

References

1. J. Darnell, H. Lodish, and D. Baltimore. Molecular Cell Biology, 2nd edition. Scientific American Books, New York, 1990.

2. W. D. Stein, Transport and Diffusion Across Cell Membranes. Academic Press, Orlando, 1986.

3. J. M. Diamond, and E. M. Wright. Annu. Rev. Physiol., 31:581, 1969.

4. C. Hansch, and A. Leo. Substituent Constants for Correlation Analysis in Chemistry and Biology. Wiley-Interscience, New York, 1979.

5. E. M. Wright, and N. Bindslev. J. Membr. Biol. 29:289, 1976.

6. M. A. Hediger, M. J. Coady, T. S. Ikeda, and E. M. Wright. Nature, 330:379, 1987.

7. E. Neher, and B. Sakmann. Nature, 260:799, 1976.

8. B. Hille, Ionic Channels of Excitable Membranes. Sinauer Associates, Sunderland, 1992.

9. C. Toyoshima and N. Unwin. J. Cell Biol., 111:2623, 1990.

10. G. M. Preston, T. P. Carroll, W. B. Guggino, and P. Agre. Science 256:385, 1992.

11. S. A. Baldwin. Biochim. Biophys. Acta 1154:17, 1993.

12. P. J. F. Henderson. Curr. Biol. 5:708, 1993.

13. M. S. Kilberg, B. R. Stevens, and D. A. Novak. Annu. Rev. Nutr. 13:137, 1993.

14. M. J. Calonge, P. Gasparini, J. Chillaron, M. Chillon, M. Gallucci, F. Rousaud, L. Zelante, X. Testar, B. Dallapiccola, F. Di Silverio, P. Barcelo, X. Estivill, A. Zorzano, V. Nunes, and M. Palacin. Nature Genet. 6:420, 1994.

15. I. M. Glynn and S. J. D. Karlish. Annu.Rev. Biochem. 59:171, 1990.

16. J. B. Lingrel and T. Kuntzweiler. J. Biol. Chem. 269:19659, 1994.

17. E. M. Wright, K. M. Hager, and E. Turk. Curr. Opin. Cell Biol. 4:696, 1992.

18. S. G. Amara and M. J. Kuhar. Annu Rev. Neurosci. 16:73, 1993.

19. E. M. Wright, B. A. Hirayama, D. D. F. Loo, E. Turk, and K. M. Hager, in Physiology

of the Gastrointestinal Tract, 3rd edition. Raven Press, New York, 1994, p. 1751.

20. S. Shafqat, B. K. Tamarappoo, M. S. Kilberg, R. S. Puranam, J. O. McNamara, A. Guadano-Ferraz, and R. T. Fremeau, Jr. J. Biol. Chem. 268:15351, 1993.

21. R. R. Kopito and H. F. Lodish. Nature 316:234, 1985.

22. D. N. Wang, W. Kuhlbrandt, V. E. Sarabia, and R. A. F. Reithmeier. EMBO J. 12: 2233, 1993.

23. M. Tse, S. Levine, C. Yun, S. Brant, J. Pouyssegur, and M. Donowitz. J. Am. Soc. Nephrol. 4:969, 1993.

24. K. D. Philipson, In: Molecular Physiology and Pharmacology of Cardiac Ion Channels and Transporters. Ed: Morad, M., Kluwer Academic Publishers, 1995.

25. W. M. Pardridge. Peptide Drug Delivery to the Brain. Raven Press, New York, 1991.

26. U. Bickel, T. Yoshikawa, E. M. Landaw, and K. F. Faull. Proc. Natl. Acad. Sci. USA 90:2618, 1993.

27. M. P. Lostao, B. A. Hirayama, D. D. F. Loo, and E. M. Wright, J. Membr. Biol., in press, 1994.

28. Y-J. Fei, Y. Kanai, S. Nussberger, V. Ganapathy, F. H. Leibach, M. F. Romero, S. K. Sing, W. F. Boron, and M. A. Hediger. Nature 368:563, 1994.

29. N. Bindslev and E. M. Wright. J. Membr. Biol. 29:265, 1976.

30. P. D. Brown, D. D. F. Loo, and E. M. Wright. J. Membr. Biol. 105:207, 1988.

31. W. A. Catterall, Science 242:50, 1988.

6
Blood-Brain Barrier Permeability: Pharmacological Implications with Special Emphasis on Peptides

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The blood-brain barrier (BBB) regulates the exchange of substances between the blood and the central nervous system (CNS). Substances exchanged include peptides and regulatory proteins. This chapter will review some factors influencing how peptides cross the BBB and other factors affecting their passage. Such background should aid in the design of therapeutic agents that are either capable of crossing the BBB themselves or that target the transport processes.

I. BBB Defined

A. Composition

The BBB may be conceptualized as those processes that combine to control the exchange of materials between the fluids of the CNS and the blood. Anatomically, these processes reside in the vasculature of the CNS, the choroid plexus, and at the circumventricular organs (CVOs). The major modification of the vasculature occurs in the spaces between the endothelial cells that line the arterioles and venules and comprise the capillary bed of the CNS [1]. Instead of having intercellular gaps that allow the production of a nourishing plasma ultrafiltrate, the adjacent endothelial cells of the CNS are held together by tight junctions. These junctions essentially eliminate the production of an ultrafiltrate in the brain. As a result, the CNS may be protected from fluid shifts, which might otherwise result in hydrocephalus or cerebellar herniation, and from circulating neurotoxins.

The lack of production of a plasma ultrafiltrate means that the CNS must rely on obtaining its nutritional requirements from the circulation by other means. Some substances are able to diffuse through the membranes that comprise the BBB [2]. Other substances, such as glucose, amino acids, and electrolytes, are transported across these membranes by means of saturable, or carrier-mediated, systems [3,4]. These nutritive and homeostatic functions of the BBB have long been known. This chapter will focus on the passage of peptides across the BBB. Because peptides perform many regulatory functions, this raises the possibility that the BBB, by controlling the exchange of regulatory substances between the CNS and blood, may also have a communicative function [5].

The endothelial cells of the CNS are modified in several ways in addition to the possession of tight junctions. For example, pinocytotic activity is decreased and surface and intracellular enzymes may present an enzymatic barrier to vulnerable substances [1,6]. An enzymatic barrier appears to be substantial for some peptides [7,8] and nonexistent for others [912].

The choroid plexus [13] lines the ventricles of the brain and are the organs of the CNS that produce most of the brain's cerebrospinal fluid (CSF). About 70% of the brain's fluids are secreted by the choroid plexus, with the remainder coming almost equally from

water produced by metabolic processes and from residual vascular leakage. The vasculature of the choroid plexus does not possess

tight junctions, but the defining outer layer of ependymal cells of the choroid plexus does. This layer, which faces and secretes the CSF, is sometimes referred to as the blood-CSF barrier. Under this terminology, the term BBB is often used to refer only to the vascular barrier. In this chapter, we will use the term BBB in its broadest sense, and use the terms endothelial barrier and ependymal barrier to refer to barriers composed of those cells. The choroid plexus are the site of many transport systems [14].

Some other areas of the brain, the CVOs, also have vasculature that is free of the BBB [1]. As with the choroid plexus, the ependymal cells that form the interface between the CVOs and the CSF have tight junctions. In addition, a functional barrier exists to diffusion between the neuropil of the CVOs and the adjacent neuropil, due either to the slow rate of diffusion with brain interstitial fluid or to a physical barrier [15,16]. Although the CVOs are unlikely to be routes for introducing bloodborne substances into areas of the CNS behind the BBB, the CVOs have neural connections to the rest of the brain and can quickly induce responses to changes in blood chemistry [17].

The BBB is, however, not absolute in its restrictive functions. Small amounts of serum proteins enter the CNS at both choroid plexus and at the vasculature. Recent evidence indicates that most of the vasculature leakage occurs not at the capillaries, but at specific sites such as the subarachnoid space/pial surface [18]. Although this evidence was gathered in studies involving high doses of horse-radish peroxidase (HRP), kinetics studies have shown that these doses of HRP do not disrupt the BBB and that the entry rates of HRP and the serum protein albumin are identical [19].

Exit, as well as entry, is important to the functions of the BBB. The CSF is largely reabsorbed into the blood through the arachnoid villi [20]. About one-third of reabsorbed brain fluid is cycled back into the periphery through a rudimentary lymphatic system; this pathway may play a key role in neuroimmune regulation [21]. Many transport systems of the BBB are directed in the brain to blood direction, constituting efflux systems. Some substances are transported both into and out of the CNS, either because their transport systems are able to shuttle bidirectionally or because opposing unidirectional transport systems occur.

The BBB has many other important aspects to its physiological functioning and new candidate features are under investigation. This complexity means that the exchange of materials between the CNS and blood can be controlled in a variety of ways, which, in turn, probably results in different physiological consequences. This chapter will address some of those aspects relevant to the exchange of peptides between the CNS and blood.

B. Operational Aspects.

The composition of the BBB underlies important aspects of its operation, especially with regard to delivery of materials to the CNS. Perhaps the most

obvious is that three major routes exist for entry of bloodborne substances into the CNS: leakage, membrane diffusion, and saturable transport.

The restrictive properties of both the endothelial and ependymal barriers are large. One way commonly used to measure this restriction is by calculation of the CSF/blood ratio of the serum protein albumin. In healthy adults, albumin seems to enter the CNS only by way of the residual leakiness of the BBB, which may be largely confined to specific areas within the brain. The CSF/blood ratio for albumin is typically 1:200 and so the CSF is often considered to be essentially free of serum proteins. However, it has not been established whether these residual amounts of serum proteins are indeed physiologically irrelevant in the CSF. For example, substances that circulate in the blood largely bound to serum proteins are often considered to be totally unbound in the CSF. This does not appear to be the case for all substances, however, as exemplified by delta sleep-inducing peptide (DSIP) [22]. Residual leakage might also allow therapeutic agents to gain significant access to the CNS. The ideal agent for such entry by this route would have a volume of distribution confined to the serum space, have little or no effect on peripheral tissues, and have a long half-time disappearance from blood because of enzymatic resistance to degradation and minimal clearance from blood.

Membrane diffusion is nonsaturable and occurs to the extent that substances are able to enter and diffuse across membranes [2]. Studies with peptides have referred to this process as transmembrane diffusion [23] to emphasize transport through membranes and not leakage around them. The rate of membrane diffusion correlates with a substance's lipid solubility and inversely with its molecular weight [2]. Charge, tertiary structure, and other factors can play major roles in special cases. Many bloodborne drugs and peptides cross the BBB to a degree large enough to produce effects on the CNS. Even modest passage by this route may affect the CNS if the substance is potent enough. For example, only about 0.02% of an intravenous (i.v.) injected dose of morphine enters the brain [24,25] but it is clearly this portion of the dose that produces changes in the electroencephalogram [26]. DSIP, thyrotropin-releasing hormone (TRH), a-melanocyte stimulating hormone (a-MSH), and cyclo(His-Pro) are peptides that have been shown to cross the BBB by a nonsaturable route [2730] and to exert actions on the CNS due to the portion of the peripherally administered dose that crosses the BBB [3034].

Saturable systems transport substances from one side of the BBB to the other [4]. These systems are selective for a substance or class of substances. For example, the hexose system transports glucose and a few other closely related hexoses [3]. A substance may be transported into the CNS, out of the CNS, or bidirectionally into and out of the CNS. Bidirectional transport of potassium may account for the relative independence between

CSF and blood levels of potassium, even though all potassium found in the CNS is derived from the

circulation [35]. Transport systems, therefore, may enhance permeability by allowing more of a substance to cross the BBB than would occur by membrane diffusion or may create the appearance of decreased permeability by transporting a substance out of the CNS after it has crossed into it. For example, brain/blood levels of many small peptides that contain an N-terminal tyrosine are lower than expected after their peripheral injection [36], in part because of a brain efflux system [37].

Saturable transport systems that do not require energy are termed facilitated diffusion and energy-requiring systems are termed active transport [38]. Facilitated diffusion, unlike membrane diffusion, is a saturable process. Many transport systems exhibit stereospecificity, although the influence of stereospecificity may vary even among the ligands for a specific system. For example, the large neutral amino acid transport system (LNAA) shows little preference for L- over D-methionine but readily differentiates between L- and D-valine [39]. The brain-to-blood efflux system for Tyr-MIF-1 and Met-enkephalin, termed peptide transport system (PTS)-1, shows stereospecificity in two ways. PTS-1 transports L- Tyr-MIF-1 but not D-Tyr-MIF-1 [40,41]. Leucine is an uncompetitive inhibitor of PTS-1, with D-leucine being about 100 times more potent than L-leucine [42].

Substances that are highly permeable to the BBB in the blood to brain direction, either because of transport systems or because of favorable characteristics for membrane diffusion, are significantly cleared from the blood during passage through the brain. Increasing the cerebral blood flow increases presentation to the brain and, therefore, increases the amount of the substance entering the brain. Such substances are termed flow-dependent, indicating that their rates of entry into the brain are largely determined by cerebral blood flow. Most endogenous peptides have entry rates too low to be affected by changes in cerebral blood flow. Peptides can, however, influence cerebral blood flow [43,44].

Brain to blood efflux can occur by way of a saturable transport system or with the reabsorption of CSF. For some substances, intracerebroventricular (i.c.v.) injection has been stated to produce concentrations in the blood identical to those after i.v. injection [20,45]. Substances injected into the brain cannot, therefore, be assumed to act only within the CNS. Other substances are sequestered by the brain after i.c.v. injection and do not appear to enter the circulation [19,46]. For these and other reasons, i.v. injection may produce a different pattern of concentrations in the brain than i.c.v. injection. In the case of thyroid hormones, some areas of the brain can be reached after i.v., but not after i.c.v., injection [47].

Other important factors affecting the distribution in brain of injected materials are rates of

diffusion within the interstitial fluid of the brain and intracranial patterns of CSF flow. Materials injected into brain tissue/interstitial fluid or into the lateral ventricle of the brain diffuse very slowly to other regions of the brain

[48]. Instead, substances tend to follow the route of CSF flow. For example, after injection into the cortical area, a substance tends to move from brain tissue towards the ventricles, through the posterior fossa, and to the arachnoid villi. A substance injected i.c.v. tends to distribute unevenly within the brain, with most of the material accumulating in the periventricular area and lesser amounts surrounding the surfaces of the brain in immediate contact with the CSF [49,50]. By contrast, substances entering the brain from the blood tend to enter evenly or, if they are flow-dependent, as a function of the rate of regional blood flow [47]. Transport systems located in the endothelial barrier tend to be evenly distributed within the brain. Pancreatic polypeptide, however, has been shown to enter the pons-medulla more than three times faster than the cerebral cortex (unpublished results). Transport into the CNS at the ependymal barriers may favor a ventricular pattern of distribution and therefore differ greatly from substances crossing at the endothelial barrier [47].

For regulatory substances such as peptides and proteins, enzymatic degradation is often a significant factor. Degradation in blood and/or clearance greatly limits the circulation time of most peptides. The BBB itself may be a significant enzymatic barrier for some peptides [7,8,51]. Enzymatic degradation within the CNS can also be quite significant for substances despite the low protein content of CSF. However, the enzymes degrading some peptides are located intracellularly rather than in the CSF and do not seem to affect passage. Perhaps for similar reasons, the CNS seems to provide a protected compartment from enzymatic degradation for some peptides. Other peptides such as b-amyloid$_{1-28}$ and PACAP28 (the 28 amino acid form of pituitary adenylate cyclase activating polypeptide) may be more quickly metabolized by the CNS than in the peripheral circulation [52,53].

Because of these considerations, brain/blood ratios, especially for areas of the brain far from the ventricles, may not be as different after i.v. as compared with i.c.v. injections, as is often assumed. In addition, difficulty exists in trying to predict brain uptake and concentration after peripheral administration for a new peptide. Even closely related substances can differ greatly in their interactions with the BBB. Peptides in particular have represented challenges to the field. It is fortunate that methods to study the permeability of the BBB to peptides are now well developed.

II. Methods for Assessment of Peptide Permeability

A. Blood-to-Brain Passage

Early work established certain principles for the passage of nonpeptides across the BBB. Methods were developed that could accurately measure the integrity

of the BBB and its disruption by such pathological events as brain tumors and hypertension [54,55]. Membrane diffusion was shown to be operable and to correlate directly with a substance's lipid solubility and inversely with its molecular weight [2,56,57]. Saturable transport systems were demonstrated and methods to measure kinetics parameters developed [3]. Methods to measure cerebral blood flow were established [58]. In vitro techniques were developed that could demonstrate binding and uptake, although not transport, by both endothelial cells and the choroid plexus [13,59,60].

Many of these techniques, however, were not sensitive enough to measure the low permeability of the BBB to peptides. For example, both morphine [56] and enkephalins [61] were reported to have a value for the brain uptake index (BUI), a classic method successfully applied in the description of BBB transport of amino acids and other substances, that cannot be differentiated from background. In the case of morphine, the low permeation was interpreted as representing penetration of the BBB at a level below the sensitivity of the BUI, while in the case of enkephalins the low permeation was taken as evidence that the BBB was essentially impermeable to peptides. The ambiguity of this double interpretation was emphasized in a recent study showing that morphine and an enkephalin analog can each alter the electroencephalogram (EEG) of rats after peripheral administration [26]. Either substance's effect on the EEG can be blocked by naltrexone, an opiate antagonist that can cross the BBB, but not by methylnaltrexone, an opiate antagonist that cannot cross the BBB. Therefore, both morphine and the enkephalin analog exert their effects on the EEG at opiate sites behind the BBB.

Newer methods with increased sensitivity have been applied to the study of peptide penetration across the BBB. The graphic method or multiple-time regression analysis [62,63] is sensitive enough to measure even the entry of vascular markers, substances with entry rates so low that they have traditionally been used to measure the vascular space of the brain. This method has been used for measuring the rates of entry of peptides, and its adaptation for measuring the permeability of the BBB to cytokines has recently been reviewed [64]. The method has also been used to measure permeability of the blood-testis barrier. There are several requirements for this method, including the need for substances that are reasonably stable in blood, since the substance is injected i.v., and so is exposed to serum proteins, interactions with erythrocytes, uptake by other organs, and so on.

Perfusion methods have been developed in which the blood flow to the carotid artery is replaced by a defined infusion [65,66]. These methodologies allow study of more pure interactions between the BBB and the substance being examined. The perfusion methods

negate factors such as enzymatic degradation in blood and protein binding since the test substance is mainly in contact with the infused vehicle. In general, the perfusion methods give entry rates that agree

almost identically with those obtained by peripheral injection methods [65]. An exception to this rule is the somatostatin analog RC-160. The entry rate of RC-160 is almost 200 times greater when measured by the brain perfusion method than when measured by multiple-time regression analysis [67]. This is probably due to protein binding of RC-160 in the circulation. Therefore, results of perfusion methods can be contrasted to those of i.v. injection methods to gain greater insight into the parameters other than BBB permeability that can affect peptide entry.

The question of complete passage of a candidate substance across the BBB as compared with its sequestration by endothelial cells has been addressed by at least three methods. One method has been recovery of material from CSF. Because there is no CSF/brain interstitial fluid barrier and because interstitial fluid flows through the ventricles before being reabsorbed into the blood, CSF represents a composite of both ependymal barrier and endothelial barrier products. The second method, capillary depletion, separates a brain homogenate into capillary-depleted and capillary-enriched fractions [68]. Appearance of a peripherally administered substance in the capillary-depleted fraction is taken as evidence that the substance has crossed the BBB completely to enter the parenchymal/interstitial space of the brain. Brain microdialysis, probably the most difficult of the three methods, has the advantage of being able to demonstrate directly the entry of peptide into the interstitial space of the brain [69]. The CSF and microdialysis methods, however, do not readily quantify rates of passage. The mathematics of multiple-time regression analysis can be applied to the capillary depletion method to quantify rates of uptake by both the capillary and parenchymal fractions.

B. CNS-to-Blood Passage

Measurement of efflux from the brain has, until recently, been difficult to assess. Because brain accumulation is a balance of influx and efflux and because brain to blood transport systems may be candidates for therapeutic manipulation just as blood to brain systems may be, study of efflux systems hold much interest. Traditionally, the most reliable method for studying efflux was the brain perfusion method [70]. Fluid was infused through the ventricular system of the brain and inflow/outflow differences were taken to represent either efflux from the CNS or uptake by brain tissue. Brain samples could be taken to differentiate between these latter two possibilities. This method is cumbersome, requires large animals for most investigators, and can only measure moderately high uptake rates. Nevertheless, this method has shown that numerous efflux systems exist for various classes of substances [70,71].

We have developed an easy method for measuring efflux [72]. Material is simply injected i.c.v. and efflux determined from straightforward equations based on either the rate of

disappearance of material from the brain or the rate of

appearance of material in the blood. The method is accurate and reliable and has been used to determine kinetics parameters for and modulators of efflux systems.

C. General Considerations

Of several difficulties in studying peptides in general and their interactions with the BBB in particular, perhaps the greatest is due to the rapid enzymatic degradation of peptides. Many peptides have a circulating half-time of minutes or even seconds. Permeability studies are usually done with radioactively labeled peptides, and the accumulation of radioactive degradation products in brain and blood must be differentiated from that of intact peptide. The use of high performance liquid chromatography (HPLC) has greatly aided this area. Based on HPLC, computation of permeability rates can be limited to those times when the radioactive label in blood and brain is still largely attached to the substance of interest. Brain perfusion methods can also be used so that degradation by blood is negated. The use of enzymatically resistant analogs [67,73,74], the study of endogenous peptides that are naturally resistant to degradation [30,75], mathematical correction to adjust data to represent only intact peptide [53,68], study of short circulation times by injection directly into the carotid artery [65], and use of antibodies that bind only intact peptide [22] are approaches that have also been used.

Because most studies use peptides that are radioactively labeled, the effect of the label on peptide/BBB interactions is of concern. The effect of a label on peptide actions probably has more to do with changes in tertiary structure, particularly those conformations relevant to membrane and binding site interactions, than on changes in primary structure. In addition, the process of labeling, which may expose the peptide to reducing agents, may also cause damage. Labeling produces changes that are difficult to predict and may diminish, enhance, or not affect biological activity. One report states that flux rates for glucose were altered by the position and type (^{3}H vs ^{14}C) of the radioactive label [76]. The brain to blood transport system for Tyr-MIF-1 seems to be unable to differentiate between peptide labeled with ^{125}I and ^{3}H [25]. The biological activity, metabolism, or disappearance rate from blood of arginine vasopressin seems to be unaffected by labeling with either tritium or radioactive iodine [7779]. Human interleukin 1a labeled with ^{125}I retains 100% biological activity, whereas interleukin 1b so labeled retains 80% of biological activity [80]. Information about the biological activity of labeled substances is unfortunately often unavailable. Perhaps one approach is to consider a radioactively labeled peptide as an analog of the original, which may or may not have altered activity, rather than to consider it in terms of being damaged or undamaged by the label. This view is supported by work that has used halogens, including iodide, to produce analogs of enkephalins [73].

The in vitro study of the BBB has been greatly advanced in recent years by the introduction of the brain microvessel endothelial cell (BMEC) model [81].

This method uses primary cultures of brain endothelial cells, grown to confluence and exhibiting many of the features of the in vivo BBB, to study characteristics of passage. Unlike the earlier in vitro models, the BMEC model can measure passage through, as well as uptake by, endothelial cells. This model has confirmed that peptides can cross the BBB bidirectionally and has demonstrated both nonsaturable and saturable permeation [11,82]. The model has also been used to study other aspects of peptide/BBB interactions such as enzymatic degradation.

III. Principles of Permeability as Applied to Peptides

A. CBF and Vascular Space

The above factors must be considered for any substance. We will now consider how they apply specifically to typical peptides. However, most peptides studied to date deviate from the general rules in at least one aspect. These deviations may play key roles in the determination of that peptide's unique behavior. The following general statements are, therefore, offered as a background to be used as an overall perspective and with which to contrast specific peptides.

In the late 1970s to early 1980s, a typical experiment would involve injection of radioactively labeled peptide into the peripheral circulation and demonstration that low amounts of radioactivity were present in samples of brain. Some authors interpreted such amounts to represent only trapping in the vascular space of the brain or leakage, while others claimed the radioactivity represented significant entry into brain. This point was resolved when experiments were conducted that coinjected radioactively labeled albumin that could act both as a vascular space marker and as a marker for measurement of residual leakage into the brain [27,83]. Another useful approach was to perfuse the brain free of blood, thus eliminating the vascular space as a source of radioactivity. These studies showed that brain/blood ratios of radioactivity for peptides significantly exceeded the vascular space and could not be accounted for by residual leakage alone. To date, no peptide has been found to have a distribution space in brain that does not at least slightly exceed the space accountable for by blood and leakage. More recent studies have shown that the typical peptide enters the brain about 100 times faster than can be accounted for by leakage [84]. Cerebral blood flow does not usually affect the degree to which circulating peptides enter the brain. This is because their rate of entry is too low to make them flow dependent.

B. Lipid Solubility

The degree to which some peptides cross the BBB correlates with their physicochemical properties, especially their lipid solubilities [36]. Peptides are usually very hydrophilic.

Nevertheless, a small percentage, typically about 2%, will

distribute into the oil phase of an experiment involving oil/aqueous phase distribution, suggesting that some bloodborne peptide could distribute into cell membranes. The unidirectional influx constant, or Ki, has been used to measure the rate at which substances cross the BBB. Peptides that enter the brain by nonsaturable processes typically have entry rates of 10^{-4} to 10^{-3} ml/g-min [84,85]. However, some endogenous peptides, such as glucagon and substance P, appear to be taken up by the brain rapidly in comparison with other peptides [36]. Molecular weight appears to play a lesser role in determining peptide permeability. In special cases, other aspects of the peptide, such as its tertiary structure or association with red cells, may act as the dominant factor in determining its permeability to membranes [114,119].

C. Saturable Transport

Many peptides have been found to have a saturable component to their permeation of the BBB. Several laboratories have described such systems by i.v. injection, i.c.v. injection, and perfusion methodologies. We have organized those described in our laboratory into a classification of peptide transport systems (PTS) [5,84]. Saturable transport systems demonstrate characteristics typical of other transport systems. They tend to transport only selected, related ligands. For example, PTS-1, the first saturable transport system to be described in the BBB for peptides, transports peptides with four to eight amino acid residues that have an N-terminal L-tyrosine [40]. However, this system does not transport all peptides that fit this description with equal avidity and, in fact, preferentially transports Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2) and methionine enkephalin (Tyr-Gly-Gly-Phe-Met).

Great difficulty accompanies attempts to determine the relationship between primary structure and affinity for transport. For example, PTS-1 does not differentiate between Tyr-MIF-1 and methionine enkephalin [86], but does transport less avidly leucine enkephalin (Tyr-Gly-Gly-Phe-Leu) and Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2), and does not transport MIF-1 (Pro-Leu-Gly-NH_2) at all [40,87,88]. PTS-1 does transport, to a small extent, oxytocin, a nine amino acid peptide that contains a tyrosine in a six-membered cyclized ring with a tail ending identical to MIF-1 [89]. Even for small peptides, it is clear that tertiary structure plays an important role in transport processes. To date, no BBB transport system for peptides has been found to be competitively inhibited by amino acids, indicating that these classes of substances are transported by different systems. A system shared by Tyr-Pro containing peptides and histidine has been found in erythrocytes [90], however.

Saturable systems may be directed in either the brain to blood or blood to brain direction. Some peptides are transported both into and out of the CNS as noted above for potassium. Bidirectional transport for nonpeptides, and possibly

for peptides, may be due to either a single system that can transport peptides in either direction or to the presence of separate systems located on either side of the BBB. Facilitated diffusion, or non-energy-requiring saturable transport, is usually bidirectional with the direction of flux being from the side of high concentration to the side of low concentration.

Some peptides may actually be transported by a family of closely related systems. For example, PTS-1, as defined with the ligand Tyr-MIF-1, has been shown to be a unidirectional brain to blood-directed system that can be inhibited by serotonin [87]. Leucine enkephalin is also transported from brain to blood but only about half of its efflux can be accounted for by the serotonin-sensitive system. In addition, leucine enkephalin is also transported into the brain [91]. At least one other system must be present in these cases to account for the blood to brain and the residual brain to blood transport. A recent review concluded that evidence existed for at least three systems capable of transporting enkephalin-related peptides, with indications that two were probably located in the choroid plexus [5]. Results suggest that the PACAP peptides, which exist in the two major forms of P38 and P27, also have different transport systems in the brain to blood and blood to brain directions [53].

Peptide transport systems are not static but can be modified by endogenous and exogenous ligands. Aluminum acts as a noncompetitive inhibitor, whereas leucine acts as an uncompetitive inhibitor of PTS-1 [92,93]. Serotonin (5HT) acts through a 5HT1-like site to inhibit transport, while 5HT antagonists stimulate transport [87]. PTS-1 appears to be under genetic control, with strains of mice with high concentrations of methionine enkephalin in the brain having high rates of transport by PTS-1 [94]. The transport rate of PTS-1 decreases with aging [95]. PTS-1 is also affected during development. Exposure of rats to morphine during the early postnatal period results in a more robust PTS-1 at the age of 21 days [96]. Such variations suggest that alterations in peptide transport systems could underlie some pathological conditions. Indeed, evidence suggests that PTS-1 may be involved in the seizures of alcohol withdrawal [97]. Manipulation of peptide transport systems might themselves be a legitimate therapeutic target.

Not all BBB binding sites for peptides are necessarily components of transport systems. Peptides have been known for almost two decades to affect the permeability of the BBB to various substances [98]. Peptides do so in at least two ways. Peptides can alter the permeability of the intact BBB to nonpeptides [98102]. Peptides may also play a role in maintaining BBB integrity. Bradykinin has been shown to regulate the permeability of tight junctions [103,104]. Leucine enkephalin and angiotensin II have been shown to increase the permeability of BMEC monolayers [105,106]. Some binding sites probably

represent receptors for peptides that are involved in regulating the metabolism of the cells that constitute the BBB. These binding sites, with their various functions, represent therapeutic targets that could alter BBB function.

D. Miscellaneous Factors and Site of Action

Factors other than peptide/BBB interactions are important in determining the amount of peptide crossing the BBB. After i.v. injection, peptides generally exhibit a volume of distribution about equal to the vascular and intercellular fluid spaces. As such, only a small percentage of the injected peptide is circulating and directly available for brain uptake. Peptides also tend to have short half-lives in the circulation. This also limits presentation to the brain. Some peptides are bound in the circulation or taken up by erythrocytes. For example, about 10% of the immunoactivity of Tyr-MIF-1 found in blood is contained in erythrocytes [90]. Some peptides form aggregates that can alter their BBB permeability [107]. As a general rule, aggregates would be expected to cross the BBB less well than monomers because of their greater molecular weights. However, aggregation by peptides may also decrease their distribution volumes, protect peptides from degradation, or limit clearance from the blood; these are all factors that would favor presentation to the BBB.

The site of action may not be the same as the site of administration. That is, a substance injected on one side of the BBB may be acting on the other side. Likewise, the site of action may not be the same as the site of effect. For example, a substance affecting brain function may be acting at sites on the blood side of the BBB. Taking these two principles together, this suggests that the site of administration and effect can both be on the same side of the BBB while the site of action is on the other. For example, peripheral administration of pancreatic polypeptide results in an effect on a peripheral tissue (pancreatic secretion). One would assume, therefore, that the site of action is peripheral. However, pancreatic polypeptide, which does not affect pancreatic cells in vitro but does enter CVOs and crosses the BBB by a saturable system, appears to act at central sites [108]. It may also be possible that a substance given i.c.v. and affecting brain function may actually be acting at a peripheral site, even though this is generally used as evidence that the substance is acting centrally. For example, it has been postulated that tumor necrosis factor given i.c.v. produces anorexia by acting at peripheral sites after entering the circulation [45]. Therefore, determination of the actual site of action of a substance can be difficult. Examples of peptides that were administered on one side of the BBB but had their sites of action on the other have been recently reviewed [109].

IV. Applications of Principles to Functional Aspects of Specific Peptides.

The above section has reviewed some of the principles affecting peptide permeability in general. This section will examine specific peptides and how these principles underlie their physiology.

Lipid solubility has been noted to be the main determinant of the extent to which many peptides cross the BBB. Although many factors ultimately determine potency, one might expect that modifying a parent peptide to make it more lipid-soluble should enhance its effects on the brain after peripheral injection. This has been shown to be true for a number of peptides including FK 33-824 [110], DSIP [32], halogenated enkephalins [73], and FMRF [111].

The presence and direction of transport systems across the BBB would be thought to play important roles in the pathophysiological and therapeutic roles of peptides. This seems to be well illustrated by a comparison of Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2) and MIF-1 (Pro-Leu-Gly-NH_2). These two peptides differ by only one amino acid [25]. However, Tyr-MIF-1 is only transported in the brain to blood direction by a system that does not recognize MIF-1, whereas MIF-1, but not Tyr-MIF-1, is quickly transported into the brain from the blood. This shows the specificity of peptide transport systems but may also account for some of the unique properties of these two peptides. The behavioral effects of MIF-1 have been studied extensively after peripheral administration. Its rapid uptake by brain has facilitated these studies. MIF-1 has shown in small clinical trials to have impressive antidepressant effects [112,113]. Again, its rapid transport into brain may underlie this effect. By contrast, Tyr-MIF-1, or at least its transport system (PTS-1) that also transports methionine enkephalin, has been linked to alcohol drinking and withdrawal [97]. PTS-1 activity levels and brain concentrations of methionine enkephalin each correlate inversely with the desire of genetically predisposed or addicted mice to drink alcohol. Reversal of suppressed PTS-1 activity after withdrawal of alcohol in addicted mice has been linked to seizure activity.

Although much attention has been focused on the permeability of the BBB to peptides as a determinant of their effects on the brain, it is clear that events in the blood also play a major role in determining how much peptide is delivered to the brain. Work with cyclo(His-Pro), the PACAP peptides, and the somatostatin analog RC-160 illustrates different aspects of circulatory effects. Cyclo(His-Pro) is a cyclized peptide with a diketopiperazine bond and would be expected to cross the BBB rapidly because of its small size and high lipophilicity. It actually crosses the BBB very slowly (Ki of about 10^{-4} ml/g-min) for reasons that are not clear but may be, in part, related to cyclization [114]. However, cyclo(His-Pro) is very stable enzymatically and eventually reaches levels in the brain sufficient to reverse ethanol narcosis [30]. Cyclo(His-Pro) is so resistant to enzymatic degradation that it has a bioavailability of about 25% after it is fed to adult mice and can reverse ethanol narcosis after oral feeding [115].

PACAP38 is larger and less lipid soluble than PACAP27. However, the Ki for PACAP38 is

significantly larger than that of PACAP27 because it is transported into the brain by a saturable system [53]. PACAP38 has a larger volume of distribution and is more rapidly cleared from the blood than PACAP27.

Therefore, when results are expressed as the percentage of i.v. administered dose entering the brain, more PACAP27 is found in the brain. When a large amount of unlabeled PACAP is given together with radioactively labeled PACAP, the rate of clearance of the labeled peptide from the blood is inhibited. Because of this enhanced presentation, the percentage of the dose administered i.v. that enters the brain increases for PACAP27. For PACAP38, however, saturation of the blood to brain transport system is more significant than the increased presentation and so less of the i.v. administered dose enters the brain. The PACAPs show the complex interplay between BBB permeability and peripheral factors that can influence the determination of how much peptide crosses the BBB. PACAP38 may yet be found to be the more potent substance because of another factor: it is much more resistant to degradation by brain enzymes.

The somatostatin analog RC-160 is a cyclized, highly lipid-soluble peptide that has antitumor and analgesic effects [116,117]. Its entry rate into brain after i.v. injection, however, is only a little faster than that of albumin [67]. One factor that may slow accumulation of bloodborne RC-160 by the brain is the presence of an efflux system, termed PTS-5. Another probable factor retarding entry is that RC-160 binds to circulating factors, because when RC-160 is presented to the brain by carotid perfusion in a serum-free medium, its rate of entry increases 200 times. This binding may be used to target RC-160 to brain tumors [118]. The entry of RC-160 into KHT sarcomas implanted into the brain was 60 times higher than into normal brain tissue. This was partially due to a more leaky blood-brain-tumor barrier, since albumin entered brain tumor tissue 10 times faster than it entered normal brain. Although the blood-brain-tumor barrier still represented a substantial barrier, the binding of RC-160 to circulating factors helped to direct it differentially towards brain tumor relative to normal brain tissue.

Entry of RC-160 into brain tumor was still six times faster than could be accounted for by increased blood-brain-tumor barrier leakage as measured by albumin. This could be explained if RC-160 had been bound to a circulating factor smaller than albumin so that the complex could have more readily crossed the blood-brain-tumor barrier. There are at least two other explanations. It may be that PTS-5, the brain to blood transport system for somatostatin-related compounds, was not operable in the tumor tissue so that accumulation would not have been decreased by this efflux system. In addition, the entry of RC-160 into brain tumor tissue and perhaps even into normal control mice of the C3H/HEN strain might have occurred partially by a saturable blood to brain transport system [118]; this system was not found in normal CD-1 mice or in Sprague-Dawley rats [67]. Taken together, the findings with RC-160 show that complex interactions of the peptide with barriers and with circulating factors can help to target the peptides to tumors.

V. Conclusions

The degree to which administered peptides enter the brain is a function of blood-brain barrier permeability, influenced by such factors as physicochemical characteristics, the presence and direction of transport systems, and peripheral effects, such as clearance, enzymatic degradation, and protein binding. Some peptides can influence factors such as cerebral blood flow and BBB integrity. An understanding of these interactions is necessary for an understanding of the actions of endogenous peptides and for the rational design of therapeutic peptide agents.

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References

1. H. Davson, K. Welch, and M. B. Segal. The Physiology and Pathophysiology of the Cerebrospinal Fluid. Churchill Livingstone, Edinburgh, 1987, p. 105.

2. H. Davson. Physiology of the Cerebrospinal Fluid. J. and A. Churchill, London, 1967, p. 82.

3. W. H. Oldendorf. Am. J. Physiol. 221:1629, 1971.

4. H. Davson, K. Welch, and M. B. Segal. The Physiology and Pathophysiology of the Cerebrospinal Fluid. Churchill Livingstone, Edinburgh, 1987, p. 247.

5. W. A. Banks and A. J. Kastin. Am. J. Physiol. 259:E1, 1990.

6. J. E. Hardebo and C. Owman. In Pathophysiology of the Blood-Brain Barrier. Edited by B. B. Johansson, C. Owman, and H. Widner. Elsevier, Amsterdam, 1990, p. 41.

7. A. Baranczyk-Kuzma and K. L. Audus. J. Cereb. Blood Flow Metab. 7:801, 1987.

8. B. T. Keller and R. T. Borchardt. Fed. Proc. 46:416, 1987.

9. J. B. M. M. Van Bree, S. Tio, A. G. De Boer, M. Danhof, J. C. Verhoef, and D. D. Breimer. Pharm. Res. 7:293, 1990.

10. T. J. Raub and C. R. Newton. J. Cell. Physiol. 149:141, 1991.

11. S. Raeissi and K. L. Audus. Ann. NY Acad. Sci. 41:848, 1989.

12. F. L. Guillot and K. L. Audus. Peptides. 12:535, 1991.

13. C. E. Johanson. In Neuromethods: The Neuronal Microenvironment. Edited by A. A.

Boulton, G. B. Baker, and W. Walz. Humana Press, Clifton, NJ, 1988, p. 33.

14. C. Nilsson, M. Lindvall-Axelsson, and C. Owman. Brain Res. Rev. 17:109, 1992.

15. B. Krisch and H. Leonhardt. Biomed. Res. 10 (Suppl 3):155, 1989.

16. B. Krisch. Cell Tissue Res. 245:101, 1986.

17. A. K. Johnson and P. M. Gross. FASEB 7:678, 1993.

18. R. D. Broadwell and M. V. Sofroniew. Exp. Neurol. 120:245, 1993.

19. W. A. Banks and R. D. Broadwell. J. Neurochem. 62:2404, 1994.

20. H. Davson, K. Welch, and M. B. Segal. The Physiology and Pathophysiology of the Cerebrospinal Fluid. Churchill Livingstone, Edinburgh, 1987, p. 485.

21. H. F. Cserr and P. M. Knopf. Immunol. Today 13:507, 1992.

22. W. A. Banks, A. J. Kastin, and D. H. Coy. Pharmacol. Biochem. Behav. 17:1009, 1982.

23. W. A. Banks and A. J. Kastin. Life Sci. 41:1319, 1987.

24. C. Advokat and A. Gulati. Brain Res. 555:251, 1991.

25. W. A. Banks and A. J. Kastin. Peptides. 15:23, 1994.

26. A. J. Kastin, M. A. Pearson, and W. A. Banks. Pharmacol. Biochem. Behav. 40: 771, 1991.

27. W. A. Banks, A. J. Kastin, and D. H. Coy. Brain Res. 301:201, 1984.

28. J. F. Wilson. Psychopharmacology 96:262, 1988.

29. B. V. Zlokovic, M. N. Lipovac, D. J. Begley, H. Davson, and Lj. Rakic. J. Neurochem. 51:252, 1988.

30. W. A. Banks, A. J. Kastin, V. Akerstrom, and J. B. Jaspan. Am. J. Physiol. 264: E723, 1993.

31. C. Okuda, H. Tanaka, and M. Miyazaki. Life Sci. 42:1181, 1988.

32. L. H. Miller, B. A. Turnbull, A. J. Kastin, and D. H. Coy. Sleep 9:80, 1986.

33. M. Holdeman and J. M. Lipton. Peptides 6:273, 1985.

34. S. T. Shih, O. Khorram, J. M. Lipton, and S. M. McCann. Am. J. Physiol. 250: R803, 1986.

35. H. Davson, K. Welch, and M. B. Segal. The Physiology and Pathophysiology of the Cerebrospinal Fluid. Churchill Livingstone, Edinburgh, 1987, p. 375.

36. W. A. Banks and A. J. Kastin. Brain Res. Bull. 15:287, 1985.

37. W. A. Banks and A. J. Kastin. Pharmacol. Biochem. Behav. 21:943, 1984.

38. P. Yeagle. The Membranes of Cells. Academic Press, Orlando, FL, 1987, p. 191.

39. W. H. Oldendorf. Am. J. Physiol. 224:967, 1973.

40. W. A. Banks, A. J. Kastin, A. J. Fischman, D. H. Coy, and S. L. Strauss. Am. J. Physiol. 251:E477, 1986.

41. W. A. Banks, A. J. Kastin, E. A. Michals, and C. M. Barrera. Brain Res. Bull. 25:589, 1990.

42. W. A. Banks and A. J. Kastin. J. Pharm. Pharmacol. 43:252, 1991.

43. J. B. Long, D. D. Rigamonti, K. Dosaka, J. M. Kraimer, and A. Martinez-Arizala. J. Pharmacol. Exp. Ther. 260:1425, 1992.

44. W. M. Armstead and C. W. Leffler. Proc. Soc. Exp. Biol. Med. 199:149, 1992.

45. R. J. Bodnar, G. W. Pasternak, P. E. Mann, D. Paul, R. Warren, and D. B. Donner. Cancer Res. 15:6280, 1989.

46. W. A. Banks, A. J. Kastin, M. B. Fasold, C. M. Barrera, and G. Augereau. Brain Res. Bull. 21:881, 1988.

47. M. B. Dratman, F. L. Crutchfield and M. B. Schoenhoff. Brain Res. 554:229, 1991.

48. H. F. Cserr. In Hydrocephalus. Edited by K. Shapiro, A. Marmarou, and H. Portnoy. Raven Press, New York, 1984, p. 59.

49. L. M. Maness, A. J. Kastin, W. A. Banks, and J. E. Zadina. Soc. Neurosci. Abstr. 17:240, 1991.

50. I. van der Ploeg, A. Cintra, N. Altiok, P. Askelöf, K. Fuxe, and B. B. Fredholm. Neuroscience 44:205, 1991.

51. D. W. Miller and R. T. Borchardt. J. Cell. Biol. 115:261a, 1991.

52. W. A. Banks, A. J. Kastin, C. M. Barrera, and L. M. Maness. Brain Res. Bull. 27:819, 1991.

53. W. A. Banks, A. J. Kastin, G. Komaki, and A. Arimura. J. Pharmacol. Exp. Ther. 267:690, 1993.

54. S. I. Rapoport. Blood-Brain Barrier in Physiology and Medicine. Raven Press, New York, 1976, p. 129.

55. V. A. Murphy and C. E. Johanson. J. Cereb. Blood Flow Metab. 5:401, 1985.

56. W. H. Oldendorf. Proc. Soc. Exp. Biol. Med. 147:813, 1974.

57. E. M. Cornford, L. D. Braun, W. H. Oldendorf, and M. A. Hill. Am. J. Physiol. 243:C161, 1982.

58. S. S. Kety. In Encyclopedia of Neuroscience. Volume I. Edited by G. Adelman. Birkhäuser, Boston, 1987, p. 206.

59. K. Brendel, E. Meezan, and E. C. Carlson. Science 185:953, 1974.

60. G. W. Goldstein, J. S. Wolinsky, J. Csejtey, and I. Diamond. J. Neurochem. 25:715, 1975.

61. E. M. Cornford, L. D. Braun, P. D. Crane, and W. H. Oldendorf. Endocrinology 103:1297, 1978.

62. R. G. Blasberg, J. D. Fenstermacher, and C. S. Patlak. J. Cereb. Blood Flow Metab. 3:8, 1983.

63. C. S. Patlak, R. G. Blasberg, and J. D. Fenstermacher. J. Cereb. Blood Flow Metab. 3:1, 1983.

64. W. A. Banks and A. J. Kastin. In: Methods in Neurosciences. Volume 16. Edited by E. B. De Souza. Academic Press, San Diego, 1993, p. 67.

65. Y. Takasato, S. I. Rapoport, and Q. R. Smith. Am. J. Physiol. 247:H484, 1984.

66. B. V. Zlokovic, D. J. Begley, B. M. Djuricic, and D. M. Mitrovic. J. Neurochem. 46:1444, 1986.

67. W. A. Banks, A. V. Schally, C. M. Barrera, M. B. Fasold, D. A. Durham, V. J. Csernus, K. Groot, and A. J. Kastin. Proc. Natl. Acad. Sci. USA 87:6762, 1990.

68. D. Triguero, J. Buciak, and W. M. Pardridge. J. Neurochem. 54:1882, 1990.

69. T. Terasaki, Y. Deguchi, H. Sato, K. Hirai, and A. Tsuji. Pharm. Res. 8:815, 1991.

70. H. Davson, J. G. Hollingsworth, M. B. Carey, and J. D. Fenstermacher. J. Neurobiol. 13:293, 1982.

71. D. J. Begley and D. G. Chain. In Peptide and Amino Acid Transport Mechanisms in the Central Nervous System. Edited by Lj. Rakic, D. J. Begley, H. Davson, and B. V. Zlokovic. Stockton Press, New York, 1988, p. 55.

72. W. A. Banks and A. J. Kastin. In Methods in Enzymology. Volume 168. Edited by P. M. Conn. Academic Press, San Diego, 1989, p. 652.

73. S. J. Weber, D. L. Greene, S. D. Sharma, H. I. Yamamura, T. H. Kramer, T. F. Burks, V. J. Hruby, L. B. Hersh, and T. P. Davis. J. Pharmacol. Exp. Ther. 259:1109, 1991.

74. S. I. Rapoport, W. A. Klee, K. D. Pettigrew, and K. Ohno. Science 207:84, 1980.

75. J. C. Verheof, H. Scholtens, E. G. Vergeer, and A. Witter. Peptides 6:467, 1985.

76. S. Wilson, J. C. Macrae, and P. J. Buttery. Res. Vet. Sci. 30:205, 1981.

77. V. T. Y. Ang and J. S. Jenkins. J. Endocrinol. 93:319, 1982.

78. L. Silver, I. L. Schwartz, C. T. O. Fong, A. F. Debons, and L. K. Dahl. J. Appl. Physiol. 16:1097, 1961.

79. C. R. W. Edwards, M. J. Kitau, T. Chard, and G. M. Besser. Br. Med. J. 3:375, 1973.

80. S. K. Dower, S. R. Kronheim, T. P. Hopp, M. Cantrel, M. Deely, S. Gillis, C. S. Henney, and D. L. Urdal. Nature 324:266, 1986.

81. K. L. Audus and R. T. Borchardt. Ann. NY Acad. Sci. 507:9, 1987.

82. W. A. Banks, K. L. Audus, and T. P. Davis. Peptides 13:1289, 1992.

83. A. J. Kastin, W. A. Banks, P. F. Castellanos, C. Nissen, and D. H. Coy. Pharmacol. Biochem. Behav. 17:1187, 1982.

84. W. A. Banks, A. J. Kastin, and C. M. Barrera. Pharm. Res. 8:1345, 1991.

85. D. J. Begley. In Handbook of Experimental Pharmacology, vol. 103, Physiology and Pharmacology of the Blood-Brain Barrier. Edited by M. W. B. Bradbury. Springer-Verlag, Berlin, 1992, p. 151.

86. W. A. Banks, A. J. Kastin, and E. A. Michals. Peptides 8:899, 1987.

87. W. A. Banks and A. J. Kastin. Psychopharmacology 98:380, 1989.

88. W. A. Banks, A. J. Kastin, and C. A. Ehrensing. J. Neurosci. Res. 35:690, 1993.

89. D. A. Durham, W. A. Banks, and A. J. Kastin. Neuroendocrinology 53:447, 1991.

90. W. A. Banks and A. J. Kastin. Biochem. Pharmacol. 40:607, 1990.

91. B. V. Zlokovic, J. B. Mackic, B. M. Djuricic, and H. Davson. J. Neurochem. 53: 1333, 1989.

92. W. A. Banks and A. J. Kastin. J. Pharmacol. Exp. Ther. 239:668, 1986.

93. W. A. Banks, A. J. Kastin, and M. B. Fasold. J. Pharmacol. Exp. Ther. 244:579, 1988.

94. W. A. Banks and A. J. Kastin. Alcohol 6:53, 1989.

95. W. A. Banks and A. J. Kastin. Neurosci. Lett. 61:171, 1985.

96. L. M. Harrison, J. E. Zadina, W. A. Banks, and A. J. Kastin. Dev. Brain Res. 75:207, 1993.

97. W. A. Banks and A. J. Kastin. In NIAAA Research Monograph 23, Alcohol and the Endocrine System. Edited by S. Zakhari. National Institutes of Health; National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD, 1993, p. 401.

98. D. Rudman and J. W. Isaacs. Endocrinology 97:1476, 1975.

99. H. Goldman and S. Murphy. Pharmacol. Biochem. Behav. 14:845, 1981.

100. P. Brust. J. Neurochem. 46:534, 1986.

101. M. Ibaragi, M. Niwa, and M. Ozaki. J. Neurochem. 53:1802, 1989.

102. D. Rudman and M. H. Kutner. Am. J. Physiol. 3:E327, 1978.

103. L. Jacques and R. Couture. Eur. J. Pharmacol. 184:9, 1990.

104. M. Wahl, A. Unterberg, A. Baethmann, and L. Schilling. J. Cereb. Blood Flow Metab. 8:621, 1988.

105. K. L. Audus, P. J. Chikhale, D. W. Miller, S. E. Thompson, and R. T. Borchardt. In Advances in Drug Research. Edited by B. Testa. Academic Press, London, 1992, p. 1.

106. F. L. Guillot and K. L. Audus. J. Cardiovasc. Pharmacol. 18:212, 1991.

107. W. A. Banks and A. J. Kastin. In Proceedings of the International Symposium on Blood Binding and Drug Transfer. Edited by J. P. Tillement, H. Eckert, E. Alben-

gres, J. Barré, P. Baumann, F. Belpaire, and M. Lemaire. Fort and Clair, Paris, 1993, p. 223.

108. D. C. Witcomb and I. L. Taylor. Am. J. Med. Sci. 304:334, 1992.

109. W. A. Banks and A. J. Kastin. Rev. Neurosci. 4:365, 1993.

110. D. Roemer, H. H. Buescher, R. C. Hill, J. Pless, W. Bauer, F. Cardinaux, A. Closse, D. Hauser, and R. Huguenin. Nature 268:547, 1977.

111. A. B. Brussaard, K. S. Kits, A. T. Maat, A. H. Mulder, and A. N. M. Schoffelmeer. Peptides 10:735, 1989.

112. R. H. Ehrensing and A. J. Kastin. Am. J. Psychiatry 135:562, 1978.

113. C. D. Van der Velde. Peptides 4:297, 1983.

114. V. Ramaswami, R. C. Haaseth, T. O. Matsunaga, V. J. Hruby, and D. F. O'Brien. Biochim. Biophys. Acta 1109:195, 1992.

115. W. A. Banks, A. J. Kastin, and J. B. Jaspan. Pharmacol. Biochem. Behav. 43:939, 1992.

116. J. I. Paz-Bouza, T. W. Redding, and A. V. Schally. Proc. Natl. Acad. Sci. USA 84:1112, 1987.

117. A. Eschalier, O. AumaÎtre, D. Ardid, J. Fialip, and P. Duchêne-Marullaz. Eur. J. Pharmacol. 199:119, 1991.

118. W. A. Banks, A. J. Kastin, S. Radulovic, F. K. Conley, D. L. Johnson, and A. V. Schally. Anti-Cancer Drugs 3:519, 1992.

119. M. Lemaire and J. P. Tillement. J. Pharm. Pharmacol. 4:715, 1982.

7
Modeling of Relationships Between Pharmacokinetics and Pharmacodynamics

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I. Introduction

Efficacy and safety of drug treatment are largely dependent on the dosing regimen. Not surprisingly, dose finding is an important aspect of drug development. For different drugs, the optimal dosage and dosing interval can be quite different. Furthermore, for a single drug the optimal dosage can differ widely between patients. Research has improved considerably our understanding of, in particular, the pharmacokinetic aspects of this variability. The values of pharmacokinetic parameters such as volume of distribution and clearance can differ widely between drugs. Furthermore, for a single drug values of pharmacokinetic parameters differ between patients. Common influential factors such as genetics, age, disease, comedication, and environmental factors have been identified in this respect.

At present, pharmacokinetic considerations still play an important role in developing dosing regimens of new drugs. Important progress has been made, however, with a more comprehensive approach based on modeling of the relationship between pharmacokinetics and pharmacodynamics. Such modeling allows the characterization and prediction of time course of drug action rather than concentration and provides a scientific basis for development of the dosing regimen. This is shown schematically in Figure 1.

Understanding the kinetics of pharmacological response in vivo is notoriously difficult due to the multifaceted nature of this endeavor. The first step in this

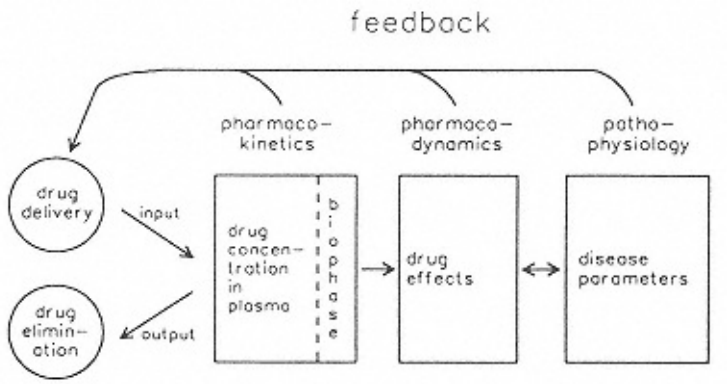

Figure 1
Schematic representation of a model for the time course of drug action. Important elements are the pharmacokinetic model, the pharmacodynamic model, and the link model. Understanding of the relationship between pharmacokinetics and pharmacodynamics provides the scientific basis for a dosing regimen.

process involves the identification of effects that truly represent the therapeutic and/or adverse effects of a drug. These effects have to be quantified in an accurate, reproducible, and sufficiently precise graded manner. Next, the insight and the mathematical strategies must be developed to relate drug and/or metabolite concentrations to the intensity of pharmacological effects. In this respect pharmacokinetic factors (i.e., distribution between blood plasma and the site of action, formation of interactive metabolites) must be taken into consideration. Finally, realistic pharmacokinetic/pharmacodynamic models must be developed that describe and predict the time course of drug action under physiological and pathological conditions.

In this chapter various aspects of modeling the time course of drug action are discussed. It is emphasized that one must not only develop a model of the time course of effect but also test its pharmacological relevance. The utilized measure of pharmacological response intensity must be pharmacologically relevant. Furthermore the predictive value of the model upon repeated administration and in different patient groups should be tested.

II. Measurement of Drug Action

For integrated pharmacokinetic/pharmacodynamic (PK/PD) investigations, the availability of realistic measures of pharmacological response intensity is essential. Such measures should meet the ideally following criteria: continuity, objectivity, sensitivity, repeatability, reproducibility, and validity [1]. For only a few drugs are measures of pharmacological response intensity available that meet each of the aforementioned criteria. This may have important implications for the modeling strategies that must be used and for the interpretation of the results. Of the various criteria, validity is an especially crucial factor.

The neuromuscular blocking agents are competitive antagonists of acetylcholine at striated muscle junctions. For these drugs neuromuscular blockade is available as an ideal measure of drug action. The technique for measurement of neuromuscular blockade (determination of the muscle or electromyographic response to motor nerve stimulation) is well established and has been applied in many PK/PD modeling studies [2].

For drugs acting on the cardiovascular system, a wide variety of different hemodynamic parameters is available that are, in principle, continuous [3]. Of the various parameters, especially blood pressure and heart rate, and, to a lesser extent, also total peripheral resistance have been popular in PK/PD modeling studies, both in healthy volunteers and in patients [4]. Some of these parameters are relevant especially with respect to side effects. On basis of blood pressure and heart rate, important information has been derived on the optimal delivery of calcium antagonists [5,6]. Whether hemodynamic variables also have predictive value with respect to the outcome of long-term

antihypertensive drug treat-

ment remains to be determined however. For antiarrhythmic drugs, effects on electrocardiographic (ECG) intervals (QRS duration, PR interval, or JT interval) have been used in many pharmacodynamic modeling studies. It has been demonstrated that prolongation of ECG intervals is, to a certain extent, correlated with the suppression of ventricular ectopic depolarizations for drugs such as quinidine, flecainide, encainide, and propafenone. The clinical relevance of ECG intervals as pharmacodynamic endpoint has nevertheless been questioned [7].

For drugs with an action on the central nervous system, measures of pharmacological response intensity are not readily available and several of the frequently used measures suffer from important limitations. Psychomotor performance tests, which are often used to assess the effects of hypnotic, sedative, and anxiolytic drugs, are prone to learning and practice effects. For this reason one should be careful in applying these tests repeatedly within the same subject. For drugs used in the treatment of depression, anxiety, and pain, rating scales are often used. Unfortunately, however, these responses are subjective and variables such as motivation and fatigue can strongly influence the results. This has important implications for the modeling approaches that can be used and for the interpretation of the results [8,9]. A final problem that one can encounter is lack of continuity. This is, for example, the case with general anesthetics. Because the responses to these drugs are quantal (response vs. no response) logistic regression has been used to derive concentration vs. response/no response relationships and estimation of the plasma concentration with 50% probability of no response (Cp_{50}) [10].

For many drugs, so-called surrogate endpoints are used in PK/PD modeling studies. Surrogate endpoints are effects that can be measured relatively easily, that exhibit most of the characteristics of ideal measures of pharmacological effect intensity (continuity, repeatability, reproducibility, sensitivity, objectivity), but of which the pharmacological relevance is often unclear. Well-known examples of surrogate endpoints are various EEG parameters for the effects of general anesthetics, benzodiazepines, and opioids. An important issue is the assessment of the pharmacological relevance. Recently, an empirical approach to the selection of EEG parameters has been proposed based on statistical considerations and prior knowledge with respect to the pharmacodynamic model [11]. A more realistic approach is to determine the correlation with effects in other in vivo and in vitro tests [1214]. This approach has been applied successfully to the benzodiazepines, barbiturates, and opioids.

The characteristic EEG changes produced by benzodiazepines are an increase in beta activity and a decrease of alpha activity. Both parameters have been used in pharmacodynamic modeling studies in humans [1517]. The pharmacological relevance of

the effect in the beta frequency band, has been determined in a series of studies in laboratory animals. The relationship between pharmacodynamic parameters derived from the EEG and those derived from in vitro receptor

binding studies and anticonvulsant effect was explored. Upon intravenous administration of 5 mg/kg of midazolam to rats, a typical increase of the amplitude in the 11.530 Hz frequency band of the EEG is observed, which returns to baseline in approximately 3 h. Analysis of this effect in conjunction with plasma concentrations results in a concentration-effect relationship that can be satisfactorily characterized on basis of the sigmoidal E_{max} pharmacodynamic model. This allows estimation of the values of the pharmacodynamic parameters E_{max}, EC_{50} and Hill factor [18]. In Figure 2 it is shown that for different benzodiazepines the values of both E_{max} and EC_{50} are quite different. A highly significant correlation between the EC_{50} obtained in the EEG model and the affinity to the benzodiazepine receptor in brain homogenate in vitro was observed (Fig. 3). Furthermore, the EC_{50} values and differences in E_{max} observed for the benzodiazepines in the EEG model also closely correlate with the pharmacodynamic parameters obtained in the pentylenetetrazol threshold model of anticonvulsant effect [19,20]. These findings justify the conclusion that, on basis of amplitudes in the beta frequency band of the EEG, indeed a realistic and pharmacologically relevant measure of the pharmacological response intensity of benzodiazepines is obtained [12].

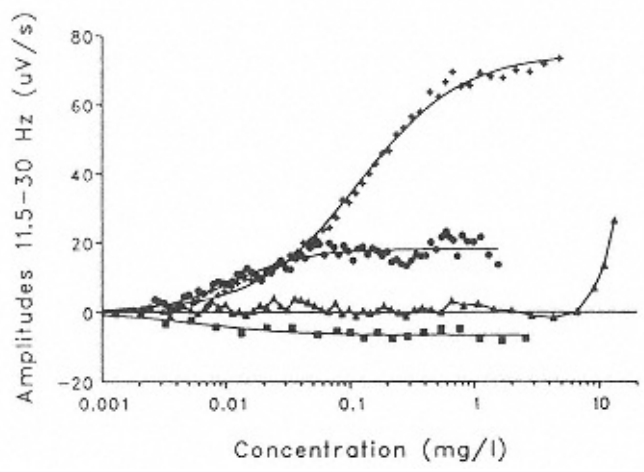

Figure 2
Concentration-EEG effect relationship in four representative rats that had received an intravenous administration of 5 mg/kg of the benzodiazepine agonist midazolam (+), 2.5 mg/kg of the partial agonist bretazenil (•), 10 mg/kg of the competitive antagonist flumazenil (▲), or 2.5 mg/kg of the inverse agonist Ro 19-4603 (▪). The change from baseline in amplitudes in the 11.530 Hz frequency band as determined by aperiodic EEG analysis was used as EEG effect measure. The sigmoidal E_{max} model was fitted to the data of midazolam, bretazenil and Ro 19-4603. (From Ref. 20.)

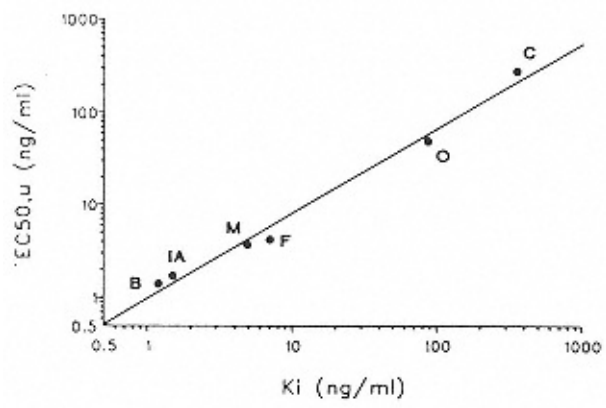

Figure 3
Correlation ($r = 0.993$, $p < 0.001$) between benzodiazepine free drug concentrations (EC50,u) producing 50% of the maximal EEG effect (change in amplitudes in the beta frequency band as determined by aperiodic EEG analysis) and affinity to the GABA-benzodiazepine receptor complex (K_i). Binding to the benzodiazepine receptor was determined on basis of displacement of [3H]flumazenil in washed brain homogenate at 37°C. (From Ref. 20.)

III. Pharmacokinetic Complexities of Pharmacodynamic Investigations in Vivo

The development of realistic pharmacokinetic/pharmacodynamic models that have predictive value beyond existing data requires that several potentially complicating pharmacokinetic factors be taken into consideration. These factors include the distribution of the drug between the blood (where generally the concentration is measured) and the site of action, protein binding, the role of interactive metabolites, and differences in disposition and pharmacodynamic properties of enantiometric drugs [21].

A. Drug Distribution

For most drugs the site of action is not identical to the blood compartment, where concentrations are generally measured. Therefore, pharmacodynamic modeling studies typically utilize concentrations at a site pharmacokinetically indistinguishable from the site of action. This can be accomplished by direct measurement of concentrations in a peripheral compartment such as the cerebrospinal fluid [22] or be relating effect intensity to concentrations in a peripheral pharmacokinetic compartment [23] or a hypothetical effect compartment [24]. It should be realized, however, that although such concentrations are di-

rectly proportional to concentrations at the biophase (i.e., the direct vicinity of the receptors), the absolute values may not be identical. Therefore changes in distribution (at the tissue level) must always be taken into consideration when studying changes in pharmacodynamics in, for example, disease states. This is illustrated in a study on the anesthetic effect of phenobarbital in experimental renal failure in which in addition to a change in brain sensitivity, a change in distribution between cerebrospinal fluid and the biophase in the brain was also observed [25].

In most pharmacodynamic investigations, drug concentrations are measured in blood plasma or serum. In plasma, drugs are bound to proteins to a varying degree and this profoundly affects the distribution. Thus in order to obtain meaningful pharmacodynamics, corrections for differences in protein binding must be made. This is illustrated in a study on the comparative pharmacodynamics of midazolam and its active metabolite a-hydroxy-midazolam [26]. In vitro data show that the affinity of the a-hydroxy metabolite is much lower than that of the parent drug. The finding that, following oral administration, concentrations of the metabolite are similar to those of the parent drug suggests that the contribution of the metabolite to the effect is negligible. In an in vivo study, however, using amplitude in the 11.530 Hz frequency band of the EEG as a pharmacodynamic endpoint, a similar potency on basis of total plasma concentrations was observed. This apparent discrepancy can be completely explained by a four-to five-fold difference in plasma protein binding. Also in situations where, for example, due to disease, alterations in plasma protein binding may have occurred, this must be taken into consideration. This is illustrated in a study on the effect of pregnancy on the antiseizure effect of phenytoin in rats [27]. When total phenytoin plasma concentrations were the focus, essentially identical results were obtained with pregnant and nonpregnant rats. After correction for the decreased protein binding, however, the concentration of free drug required for adequate seizure control was shown to be higher in pregnant than in nonpregnant rats. A special situation arises, finally, when plasma protein binding is saturable in the relevant concentration range. For disopyramide, nonlinearity in the plasma protein binding must be incorporated in the modeling in order to obtain meaningful pharmacodynamic results [28].

For several drugs marked arteriovenous concentration differences have been reported and it has been postulated that the high uptake of drug by a relatively poorly perfused sampling tissue is responsible for this phenomenon [29,30]. As a result, quite different pharmacokinetic/pharmacodynamic relationships can be observed, depending on the sampling site. This is illustrated in a study on the pharmacodynamics of thiopental using EEG effects as a pharmacodynamic endpoint [31]. For this drug, significant arteriovenous concentration differences exist. When arterial blood samples are used, a high degree of

hysteresis is observed and, therefore, concentrations in a hypothetical effect compartment must be es-

timated to derive concentration-EEG effect relationships. When using venous sampling, the EEG effects can be related directly to the plasma concentrations. An important question is whether in pharmacodynamic investigations, arterial or venous blood concentrations should be preferred. It is often stated that since the arterial plasma concentration carries the drug to the site of action, arterial plasma concentrations should be preferred. To date, however, no studies have been published that show that on basis of arterial blood concentrations indeed more realistic pharmacodynamic information is obtained. A relative disadvantage of venous sampling is that, especially for drugs that distribute rapidly to the site of action, proteresis (clockwise hysteresis) may occur. This makes estimation of hypothetical effect compartment concentrations more difficult. Furthermore, by the use of venous blood concentrations variability in the pharmacodynamic parameter estimates may be increased. Improved precision may justify the collection of arterial blood samples, especially in situations in which complex modeling is needed (e.g., acute tolerance development).

B. Active Metabolites

Many drugs are converted in the body into metabolites. These metabolites can, in principle, possess agonistic or antagonistic pharmacological activity. Formation of metabolites can therefore significantly affect or even be the main determinant of the time course of the pharmacological response [32]. Also it can explain why the pharmacological response can be different upon different routes of administration. For quinidine, using electrocardiographic effect parameters and systolic time intervals, it was found that the slope of the linear concentration-effect relationship is different upon oral vs. intravenous administration [33]. Likewise for midazolam, a shift of the concentration-effect relationship to lower concentrations was observed upon oral administration (Fig. 4) [34]. In both cases active metabolites formed during first-pass contribute to the effects following oral administration. Following intravenous administration this contribution is much less. Pharmacodynamic interaction models are needed to predict the time course of effects of these drugs following oral administration (see Section V).

In order to incorporate interactive metabolites into modeling of the kinetics of pharmacological response of a drug, independent knowledge on the pharmacological activity of the metabolites can be helpful. Such information can be obtained in the following ways: by direct administration and analysis of the effects, by analysis of the pharmacological effects following different rates and routes of administration, and by the utilization of radioreceptor assays [21]. Direct administration of metabolites has frequently been used [21], and has the advantage that detailed information on the pharmacodynamic properties (e.g., potency, intrinsic activity) can be obtained [26].

Potential antagonistic action of metabolites cannot be determined in this way, however. Furthermore, the ap-

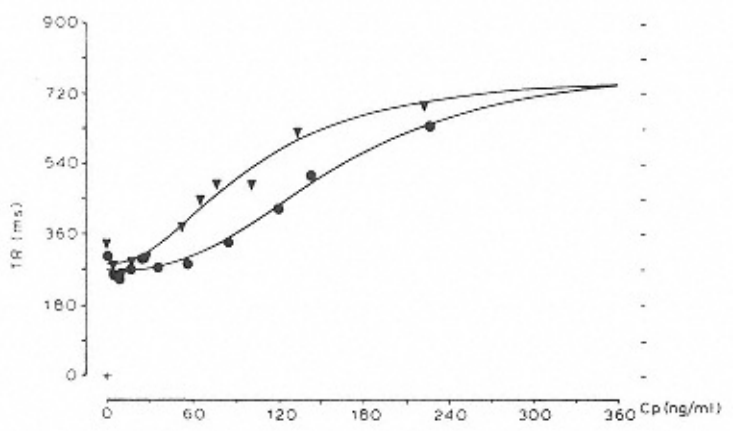

Figure 4
Concentration-pharmacological response relationship of midazolam following oral (▼) and intravenous • administration. The pharmacological response was determined as the reaction time (TR) after an acoustic stimulus. The difference in concentration-pharmacological response relationship can be accounted for by the formation of significant amounts of the active metabolite a-hydroxy-midazolam following oral administration. (From Ref. 34.)

proach can only be applied to identified metabolites that can be synthesized in sufficient quantities and can be administered safely to humans. Indirect assessment of the pharmacological activity of metabolites, by varying the rate and route of administration, has been applied in model studies with midazolam and heptabarbital [18,35]. The approach is attractive, because it is possible to screen for the presence of active metabolites without the need to have the metabolites available, to administer them, or even to know their chemical structure. Also information can be obtained on both the role of agonistic and antagonistic actions of metabolites. Although not strictly necessary, the measurement of concentrations of known metabolites in this type of investigations does offer interesting perspectives. By knowing these concentrations it is, in principle, possible to derive, on the basis of a suitable interaction model, the pharmacodynamic properties of the metabolite. Thus far, however, no formal studies have appeared in which this approach has been applied.

Radioreceptor assays (RRAs) are based on the competition for receptors between a radioactively labeled ligand and the compound to be analyzed. Both parent compound and active metabolites can therefore be detected by these relatively simple and rapid techniques. By comparison of RRA results to those obtained with specific assays procedures (gas-liquid chromatography ([GLC], high-performance liquid chromatography [HPLC]), the formation of interactive

metabolites can be detected [36]. Radioreceptor assays have been developed for benzodiazepines, opioid analgesics, and neuroleptics [37].

C. Enantiomers.

Many pharmacologically active compounds are optically active and are usually administered as racemate. The stereoisomers, however, can differ in both their pharmacokinetic and pharmacodynamic characteristics, which imposes similar problems on the interpretation of pharmacodynamic data as with interactive metabolites: the observed pharmacological response is the result of two or more compounds with different pharmacological properties on the same pharmacological system. The differences in pharmacokinetic behavior of enantiomers have been the subject of many reviews [3842]. In many instances the clearance of one enantiomer is much higher relative to the other. In addition, differences in distribution have been demonstrated due to stereoselective differences in plasma protein binding.

In the literature are numerous examples of pharmacodynamic investigations in which stereoselectivity in disposition is critical to interpretation of the results. For verapamil, using total concentrations of both enantiomers, a marked shift in the concentration-effect relationship to higher concentrations was observed upon oral vs. intravenous administration (Fig. 5). This can be explained in part

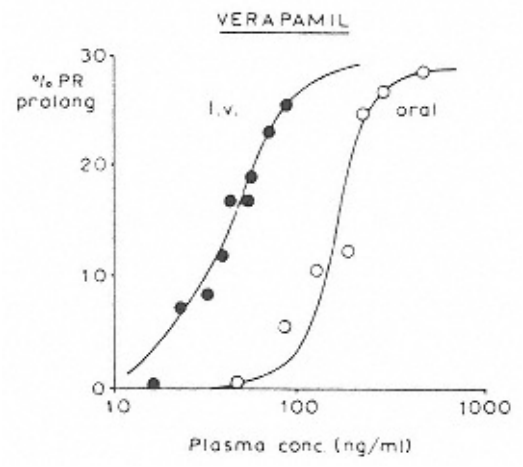

Figure 5
Effect of route of administration on the plasma concentration- pharmacological response relationship of verapamil, based on total concentrations of both enatiomers. The shift in the concentration-effect relationship can be explained by stereoselective first-pass elimination resulting in a relative enrichment of the least potent enantiomer. (From Refs. 42

and 43.)

by stereoselectivity in the first-pass effect, resulting in a relative enrichment of the less active (+) enantiomer [43,44]. For propranolol, using exercise-induced tachycardia as a pharmacodynamic measure, the total concentration-effect relationship was found to be shifted to lower concentrations upon oral administration, which can be attributed in part to the first-pass effect being selective for the less active enantiomer [45]. Also in studies on the effect of genetic factors, disease, and drug-drug interactions it is essential that stereoselectivity in disposition be taken into account, as was demonstrated for metoprolol in phenotypically poor metabolizers of debrisoquine [46], verapamil in patients with liver disease [45,46], and in a study on the interaction between phenylbutazone and warfarin [49].

IV. Pharmacodynamics and Pharmacodynamic Models

Pharmacodynamics comprises the processes of the drug-receptor interaction, postreceptor events (= effectuation: i.e., effects on ion channels, formation of second messengers, etc.) triggering the ultimate pharmacological response, and the homeostatic mechanisms that may be operative (Fig. 6). Each of these pro-

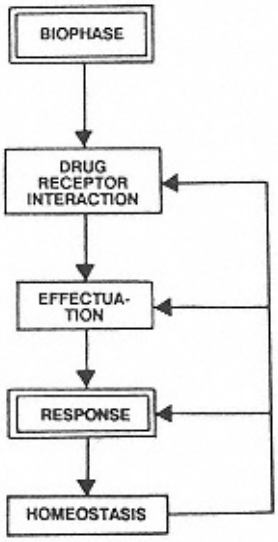

Figure 6
Schematic representation of the processes in the pharmacodynamic phase of drug action. Each of these processes requires time and can therefore be a determinant of the time course of drug action in vivo. (From Ref. 50.)

cesses requires time and this may affect the time course of drug action in vivo. For most drugs there is a rapid equilibrium between the concentration at the site of action and the receptor. However, binding to a receptor may also be irreversible, as is the case with, for example, certain anticancer drugs. Postreceptor events may be rapid (e.g., the neuromuscular blocking drugs) or slow (coumarin anticoagulants, corticosteroids, antidepressants). Finally, homeostatic mechanisms may be operative (e.g., tolerance/sensitization) that may have time constants varying from seconds to days or even weeks. Thus, in modeling the time course of drug action in vivo, the rate constants of processes in the pharmacodynamic phase of drug action must be taken into account [50].

Pharmacodynamics is often characterized on the basis of time-independent (steady-state) drug concentration-pharmacological response relationships. One of the most well known pharmacodynamic models is the Emax model. The mathematical form of this model is:

$$E = E_0 + \frac{E_{max} \cdot C}{EC_{50} + C} \quad (1)$$

where E is the pharmacological effect intensity, E0 is the pharmacological response intensity when the drug concentration is zero, Emax is the maximum response the drug can produce, C is the drug concentration, and EC50 is the concentration that produces 50% of the maximum response. In many instances, the Emax model needs to be extended in order to accommodate differences in the steepness of the concentration-effect relationship. For that purpose, the slope factor n is incorporated:

$$E = E_0 + \frac{E_{max} \cdot C^n}{EC_{50}^n + C^n} \quad (2)$$

This model is often referred to as the sigmoid Emax model. The additional parameter n (Hill factor), makes the curve steeper when it is greater than 1 and more shallow when less than 1. The change in concentration required for effect to change from 20% to 80% of Emax is 16 fold when n is 1 but is only fourfold when n is 2. Values of n greater than 5 approximate a quantal response: there is a threshold concentration below which no drug effect occurs, an almost full response is observed at concentrations just above this threshold. Sometimes simplifications of the (sigmoidal) Emax model may be used to characterize the relationship between concentration and response. When drug concentrations are well below the EC50, the pharmacodynamics may adequately be described by the linear model:

$$E = E_0 + \frac{E_{max}}{EC_{50}} \cdot C \quad (3)$$

In this case the ratio of E_{max} and EC_{50} is the slope of a straight line with intercept E_0. In the concentration range producing between 20 and 80% of the maximal effect, the relationship between effect and the logarithm of the concentration is linear. Application of the log-linear model is associated with serious limitations: it does not predict a maximum response or a zero response. Its use is therefore not recommended. In certain instances the relationship between drug concentration and pharmacological effect intensity is more complex and different models have to be used. An example is the effect of thiopental and other barbiturates on the EEG. Here a biphasic effect is observed: increase in EEG effect at low concentration followed by a decrease at higher concentration. A descriptive model has been proposed to characterize the concentration-effect relationship [51]. This is shown in Figure 7.

The pharmacodynamic models described above are particularly useful in modeling reversible pharmacological effect when the effects in the pharmacodynamic phase are rapid. A rate constant K_{eo} can then be incorporated in the model to account for the delay between drug concentration in plasma and the time course of the pharmacological response. However, when the processes in the pharmacodynamic phase are relatively slow or irreversible, other modeling approaches may be required. This is discussed in Section V.

V. Models of the Time Course of Drug Action

In recent years several models have been developed to correlate the time course of pharmacological effect intensity to the concentration vs. time profile in the body. Integrated pharmacokinetic-pharmacodynamic models can be relatively simple if the effect of the drug is direct, concentrations in blood-plasma are in rapid equilibrium with the site of action, and the processes in the pharmacodynamic phase of drugs action (biochemical and receptor-mediated) are fast. On the other hand, if one or more of these criteria are not met, models can become extremely complex. In this situation often several alternative models are available to describe the data and a detailed understanding of the various processes determining the time course of drug action may be required to develop a realistic model with adequate predictive value [52].

A. Direct Effects

In pharmacodynamic investigations in vivo, often a delay of the pharmacological effect vs. time profile relative to the plasma concentration is observed. In this situation often a model composed of a classic compartmental pharmacokinetic model, a hypothetical effect compartment, and a classic pharmacodynamic model is used to characterize the data, by which the equilibration with the effect compartment serves to account for the temporal

delay between plasma concen-

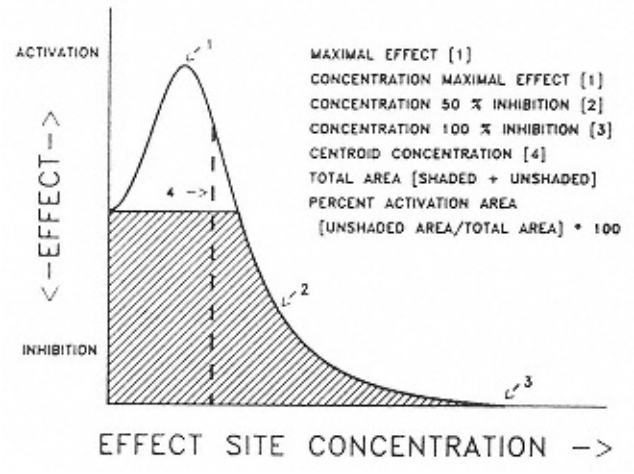

Figure 7
Empirical model to describe the biphasic relationship between concentration and EEG effect of thiopental in rats. The various descriptors not only quantitate the morphology of the concentration-effect relationship but also reflect the potency of the anesthetic. (From Ref. 51.)

tration and effect. The effect compartment is linked to the plasma compartment by a first-order rate constant k_{1e} and with a rate constant for drug losses, k_{eo} (Fig. 8).

The rate of change of drug concentration in the hypothetical effect compartment can be expressed by the following differential equation:

$$\frac{dCe}{dt} = k_{1e}Cp - k_{eo}Ce \quad (4)$$

where Cp is the measured drug concentration in the central compartment of the pharmacokinetic model (mostly plasma concentration) and Ce is the drug concentration in the hypothetical effect compartment. It is assumed that the effect site receives a negligible mass of drug. As a consequence, the effect compartment does not affect the pharmacokinetics of the drug. The effect compartment concentration is a hypothetical concentration and can never be measured. Therefore, it is assumed that in steady state the effect compartment concentration equals the plasma concentration. This will ensure that the effect compartment concentration actually reflects the steady-state plasma concentration that causes the same effect as that effect compartment concentration. Thus, the effect compartment concentration vs. effect relationship represents the steady-state plasma concentration vs. effect relationship. From the assumption that in steady state

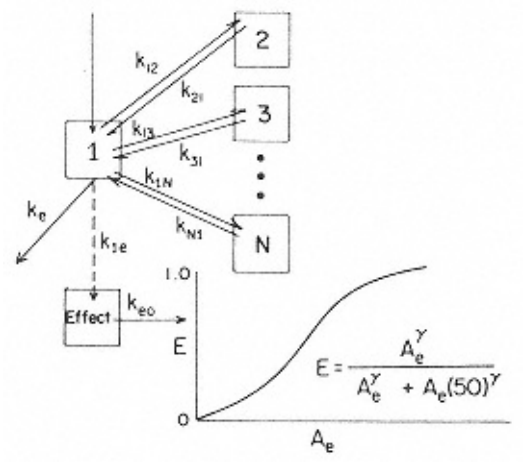

Figure 8
The effect compartment model for modelling of direct effects. In this model pharmacological effect intensity is directly related to concentrations in a hypothetical effect compartment, which receives its input from the central compartment. Equilibration with the effect compartment, characterized by the rate constant k_{eo}, accounts for the temporal delay between plasma concentration and effect. (From Ref. 55.)

the effect site concentration equals the plasma concentration it follows that k_{1e} equals k_{eo}. Thus the hypothetical effect site concentration can be calculated from the following differential equation

$$\frac{dCe}{dt} = k_{eo}\,(Cp - Ce) \quad (5)$$

or the following equivalent convolution equation:

$$Ce = keoe\text{-}keot * Cp \quad (6)$$

where the * operator represents convolution.

The approach of pharmacokinetic/pharmacodynamic modeling was first successfully applied to D-tubocurarine [53]. In this instance, a model was used that is presently often referred to as a full parametric model. This type of modeling requires prior knowledge of the pharmacokinetic model to describe the plasma concentration vs. time profile, the pharmacodynamic model to describe the concentration-effect relationship, and the link model that characterizes the equilibration kinetics between blood plasma and the hypothetical effect compartment. Often the sigmoid E_{max} model or one of its simplifications (i.e., the E_{max} or the linear model) is used to describe the concentration-effect relationship. Furthermore the equilibration with the hypothetical effect compartment is almost always

considered to be a first-order process, characterized by the first-order rate constant k_{eo}. In the model only the parent drug is pharmacologically active (i.e., no active metabolites are formed). Furthermore it is assumed that the effect intensity depends only on the current concentration (i.e., no tolerance or sensitization occurs). Derivation of the equations that characterize the time course of the pharmacological response in this type of full parametric models is relatively straightforward [54]. Functions describing the drug concentration at the effect site over time are easily obtained by substituting the pharmacokinetic model (describing the time course of plasma drug concentrations) in Equations (5) or (6). For instance, if the disposition of a drug in the plasma can be described by a one-compartment open model after a bolus intravenous input of a dose D, Equations (5) and (6) will yield the following solution:

$$Ce = \frac{k_{eo} D}{V_1(k_{eo} - k)} (e^{-kt} - e^{-k_{eo}t}) \quad (7)$$

where k is the elimination rate constant of drug loss from the plasma and V_1 is the volume of the central compartment. Substitution of this expression into a pharmacodynamic model (i.e., the sigmoidal E_{max} model) results in an equation for the time course of the pharmacological response intensity. Nonlinear regression of the time course of drug action on basis of one of these equations allows estimation of the value of k_{eo} as well as the values of the pharmacodynamic parameters (e.g., E_{max}, EC_{50} and n).

In addition to the full parametric methods, semiparametric and nonparametric approaches to effect compartment modeling have been proposed. These approaches differ mainly with respect to the prior knowledge required. In the semiparametric approach, the value of k_{eo} is estimated as the value that causes the hysteresis curve (concentration vs. effect intensity connected in time order) to collapse to a single curve that represents the steady-state concentration-effect relationship. Thus the value of k_{eo} is estimated without postulation of a particular parametric model for the concentration-effect relationship. The method has the important advantage that it allows inspection of the concentration-effect relationship, before selecting a particular pharmacodynamic model. In this way the risk of model misspecification is minimized [55]. Finally, an approach for pharmacodynamic modeling has been proposed that represents both pharmacokinetics and pharmacodynamics nonparametrically, but retains a parametric link model [56]. This approach may be of value in exploratory data analysis and in situations where the pharmacokinetics cannot be easily characterized on basis of a compartmental pharmacokinetic model (e.g., sustained release preparations).

In most pharmacodynamic modeling studies the rate of equilibration with the hypothetical effect compartment is considered a first-order process. However an algorithm and

computer program COLAPS has been proposed [57] that allows different forms of the link model (monoexponential vs. polyexponential link

models). In a study with amobarbital, using quantitative EEG parameters as pharmacodynamic endpoint, it was demonstrated that a biexponential link model results in a considerable improvement of the hysteresis. Furthermore, the form of the concentration-effect relationship and the estimates of the pharmacodynamic parameters are quite different. This is illustrated in Figure 9 [58]. Recently, an algorithm and computer program, ATRACT, has become available that uses a different approach for minimalization of hysteresis [59].

An important question in using effect compartment models relates to the physiological meaning of pharmacodynamic parameter EC_{50} and the rate constant k_{eo}. In compartmental and physiological terms, the effect compartment can be interpreted as a drug distribution compartment. The values of concentrations in this compartment cannot be measured experimentally and are therefore assumed to be equal to the measured drug concentration in steady state. Therefore, the value of the EC_{50} obtained by effect compartment modeling represents the steady-state plasma concentration producing 50% of E_{max}. The rate constant k_{eo} can be considered as pharmacokinetic distribution rate. This is appropropriate in situations in which the effect occurs instantaneously once molecules reach the site of action. In this particular situation, the physiological determinants of k_{eo} can be thought of in terms of the delivery rate to the tissue (i.e., blood flow and apparent volume of distribution of the tissue) [60]. The neuromuscular blocking drugs are an example of a class of drugs in which k_{eo} reflects the delivery of the drug to the site of action at the neuromuscular junction. For D-tubocurarine the value of k_{eo} is lower with halothane anesthesia relative to nitrous oxide-narcotic anesthesia. This can be explained by a reduction in skeletal blood flow under the influence of halothane [61]. For most drugs, the meaning of the value of k_{eo} in physiological terms is not understood. Rate processes in the pharmacodynamic phase of drug action can also be an important determinant.

B. Indirect and Irreversible Effects

For certain drugs the delay between drug plasma concentrations and pharmacological effect intensity is largely determined by a slowly developed effect rather than by slow distribution to the site of action. For these drugs effect compartment models are not appropriate and so-called physiological substance models should be used. Such models have been described for the coumarin anticoagulants, the corticosteroids, and nonsteroidal anti-inflammatory drugs (NSAIDS).

The anticoagulant effect of warfarin and its analogues results from concentration-dependent inhibition of vitamin K-dependent synthesis of the prothrombin complex of coagulation factors [62]. As a result, the rate of elimination of the prothrombin complex

rather than distribution to the site of action is the major

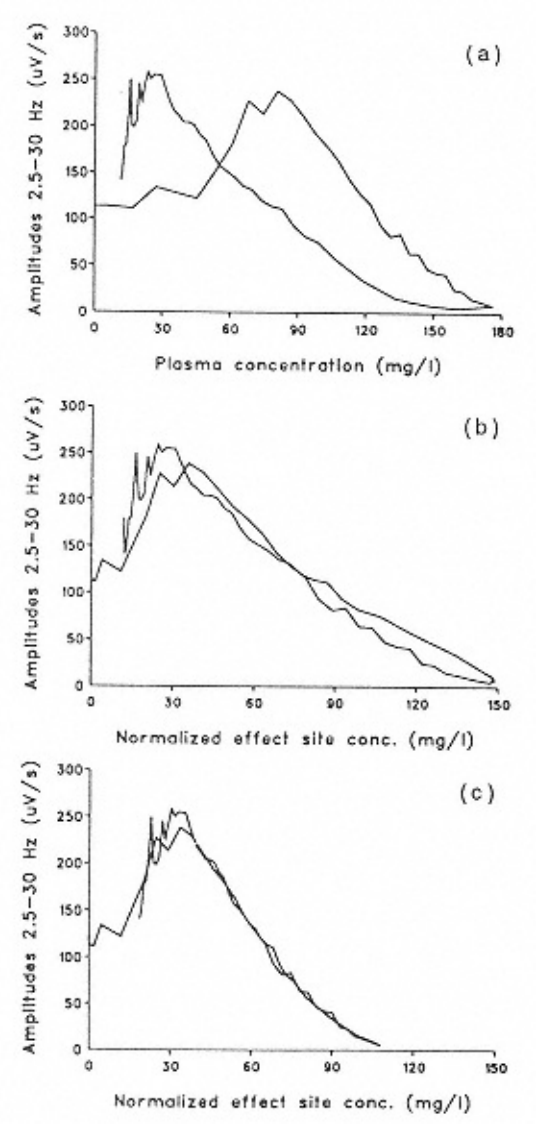

Figure 9
Pharmacodynamics of amobarbital in rats. Plasma concentration vs. EEG effect hysteresis curves (a) and normalized effect site concentration versus EEG effect curves after hysteresis minimalization with a monoexponential (b) and a biexponential (c) link model. The concentration-effect relationship is biphasic (increase of EEG effect at low concentration and decrease of EEG effect at higher concentration). It appears that much better hysteresis minimalization is obtained with the biexponential link model. The shape of the obtained concentration-effect relationship is also different. (From Ref. 58.)

determinant of the delay in the onset of the effect. Essential elements in modeling the time course of anticoagulant effect are a) a pharmacodynamic model for the effect on the synthesis of vitamin K-dependent clotting factors (expressed as prothrombin complex activity [PCA] and b) a physiological substance model for the rates of synthesis and degradation of vitamin K dependent clotting factors again expressed as PCA. The time course of the anticoagulant effect warfarin, expressed as PCA, can be described by the solution to:

$$\frac{dPCA}{dt} = R_{syn} \cdot f(W) - k_{deg} \cdot PCA \quad (8)$$

where R_{syn} is the synthesis rate of clotting factor activity, k_{deg} is the degradation rate constant for clotting factor activity, and f(W) is the PD model that predicts the percentage change in synthesis rate as a function of anticoagulant concentration. Several models for f(W) have been evaluated (e.g., linear, log-linear, Emax, and sigmoid Emax models). The synthesis rate is assumed to have a constant value independent of the changes in prothrombin activity in time, thus

$$R_{syn} = R^0_{syn} = k_{deg} \cdot PCA_0 \quad (9)$$

from which it follows that the change in PCA can be described by:

$$R_{syn} = R^0_{syn} = k_{deg} \cdot PCA_0 \quad (10)$$

where $R0_{syn}$ is the synthesis rate at baseline, and PCA_0 is the prothrombin complex activity in the absence of anticoagulant [60,6264].

The pharmacodynamics of the nuclear or gene-mediated effects of corticosteroids are extremely complex and involve many different steps [65]. Recently a model for the induction of the liver protein tyrosine aminotransferase (TAT) as an easily quantifiable, gene-mediated effect of corticosteroid in vivo has been proposed (Fig. 10). In the model the major rate-limiting steps in the kinetics of the gene-mediated effect (i.e., the pharmacokinetics, the steroid receptor on-off rates, receptor loss and return to hepatic cytosol, and the induction and degradation of an enzyme) are mathematically described. In total the model consists of six differential equations, with a mixture of physiological parameters and purely empirical terms. The drug-receptor-DNA interactions are described by:

$$\frac{d(R)}{dt} = -k_{on} \cdot D.R. + k_{off} \cdot DR + DRN \quad (11)$$

$$\frac{dDR}{dt} = k_{on} \cdot D.R. - (k_{off} + k_{DNA}) \cdot DR \quad (12)$$

$$\frac{dDRN}{dt} = (k_{DNA} \cdot DR - DRN)/\tau \quad (13)$$

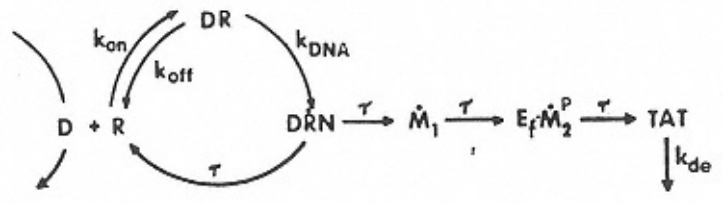

Figure 10
Pharmacodynamic model for gene-mediated corticosteroid action. D, hepatic cytosol drug concentration; R, hepatic receptor concentration; DR, concentration of the drug-receptor complex; M_1 and M_2, intermediary transfer compartments; TAT, hepatic tyrosine aminotransferase activity; k_{on} and k_{off}, rate constants for drug receptor association and dissociation; k_{DNA}, rate constant for DR binding to DNA; t, transfer time parameter; E_f, efficiency factor; P, power term; k_{de} rate constant for degradation of TAT. (From Ref. 66.)

where *D* is the drug concentration at the hepatic cystosol receptor site, R is the free hepatic receptor concentration, DR is the drug-receptor complex, DRN the drug-receptor-DNA complex concentration, k_{on} and k_{off} the rate constants for drug-receptor association and dissociation, k_{DNA} the rate constant for DR binding to DNA, and t a transfer time parameter. Two additional, empirical steps describe the rate of change between DNA binding and enzyme induction:

$$\frac{dM_1}{dt} = (DRN - M_1)/\tau \quad (14)$$

$$\frac{dM_2}{dt} = (M_1 - -M_2)/\tau \quad (15)$$

in which M_1 and M_2 are intermediary transfer compartments. The ultimate effect is described by:

$$\frac{d(TAT)}{dt} = E_f M - k_{de} \cdot TAT \quad (16)$$

where E_f is an efficacy factor, p an empirical power term, and k_{de} a rate constant for the degradation of TAT. This model has been applied successfully to characterize the time lag for TAT activity following the administration of prednisolone in rats (Fig. 11) [66]. The model has been found useful to predict the time course of effect following different dosage regimens [67] and to study the effects of disease on the kinetic-dynamic determinants of corticosteroid action [68].

Recently four general but structurally different models for characterizing indirect pharmacodynamic responses have been developed and compared [69].

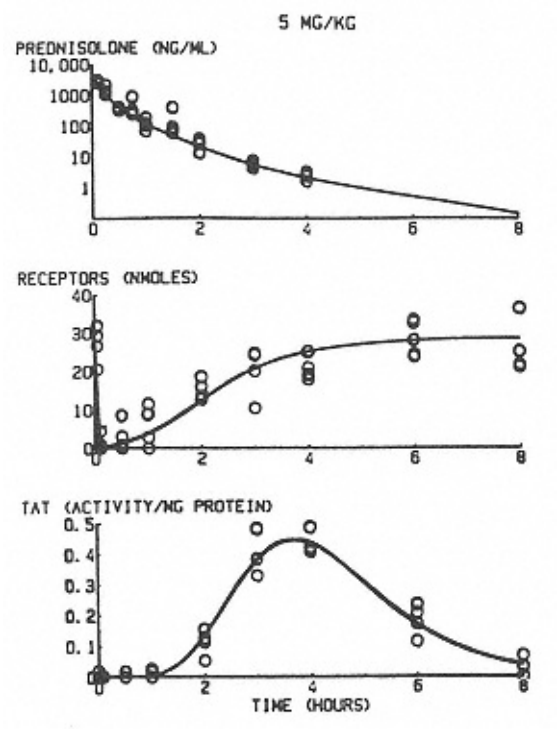

Figure 11
Modeling of the gene-mediated effect of corticosteroids in rats. Shown is the time course of the measured and predicted plasma prednisolone concentrations, hepatic cytosol receptor concentrations, and hepatic TAT activity after a 5 mg/kg intravenous dose of prednisolone (From Ref. 66.)

These models are based on drug effects (inhibition or stimulation) on the factors controlling either the input or the dissipation of the drug response. These models provide a useful starting point for the evaluation of pharmacological effects in which the site of action precedes or follows the measured response variable.

Antineoplastic agents often cause irreversible effects (i.e., cell death) by irreversible or covalent incorporation into a metabolic pathway of the cell [70]. Pharmacodynamic models for the actions of antineoplastic drugs have been postulated in which a differentiation is made between cell cycle-nonspecific effects and cell cycle-specific effects [71,72]. These models are shown in Figure 12. For cell cycle-nonspecific chemotherapeutic agents, the rate of change in the concentration of target (tumor) cells can be described on basis of the following

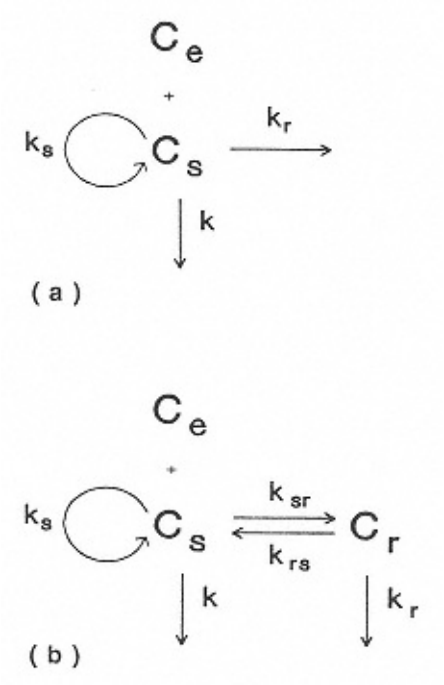

Figure 12
Pharmacodynamic models for the cell cycle nonspecific and cell cycle-specific effects of chemotherapeutic agents. C_e, drug concentration at the site of action; C_s, density of cells that are sensitive to the drug; k, drug-induced irreversible cell death rate constant; k_s, natural cell proliferation rate constant; k_r, rate constant of physiological degradation of cells. In model (a) all tumor cells are sensitive to the drug and therefore C_s is equal to the total density of the cells. In model (b) the sensitivity is dependent on the specific phase of the cell cycle. Thus in this model in principle two populations of cells exist, sensitive cells and insensitive cells. In this model C_r represents the density of insensitive cells and k_{rs} and k_{sr} are the interconversion rate constants between the two cell types. (From Ref. 72.)

equation:

$$\frac{dC_s}{dt} = k_s \cdot C_s - k_r \cdot C_s - k \cdot C_s \cdot C_e \quad (17)$$

where C_e is the concentration at the effect site, C_s is the density of cells, k the drug-

induced irreversible cell death rate constant, k_S the natural cell proliferation

rate constant, and kr the rate constant of the physiological degradation of cells. On basis of this model useful equations can be derived for the relationship between drug exposure (dose) and the survival of tumor cells. Integration of Equation (17) yields:

$$\ln \frac{C_x}{C_o} = (k_s - k_r)t - k \int_0^t C_e \, dt \quad (18)$$

where Co is the concentration of cells before the initiation of therapy. This equation shows that the logarithm of the survival fraction is directly proportional to the exposure of drug (area under the curve [AUC]) at the site of action. When the total administered dose of the drug has been eliminated then

$$\ln \frac{C_x}{C_o} = (k_s - k_r)t - k_p \int_0^\infty C_p \, dt = (k_s - k_r)t - \frac{k_p F}{Cl} D \quad (19)$$

where D is the administered dose, F is the bioavailability, and Cl is the clearance of the drug. This equation shows that survival of tumor cells is directly proportional to the administered dose. This model has been applied successfully in a study in mice in which the effect of intraperitoneal administration of single doses of cyclophosphamide on the survival of chimera spleen cell and osteosarcoma cells was determined [68]. The results of this study have been summarized in Figure 13.

The equations required to characterize the pharmacodynamics of cell cycle-specific chemotherapeutic agents are more complex. In this situation there are two populations of tumor cells, sensitive and insensitive cells, and therefore also the interconversion between these two cell types must be taken into consideration (Fig. 12). The full equations for modeling the time course of drug effect can be found in the literature [71]. The model has been applied in a study on the effect of vinblastine [72].

C. Combined Drug Action

The situation of combined drug action arises when two or more pharmacologically active compounds exert an effect at a certain biological system and when a single drug acts simultaneously at two different receptor sites. Different modeling strategies are required in these situations [73].

In many instances drug effects are the result of the combined action of two or more chemical entities: when two or more drugs are given in combination, when a drug is converted into an interactive metabolite, and in the situation where, for an enantiomeric drug, a racemate of the two enantiomers is administered or metabolic interconversion occurs. Effects may also result from the combined action of a drug and an endogenous compound. In these situations,

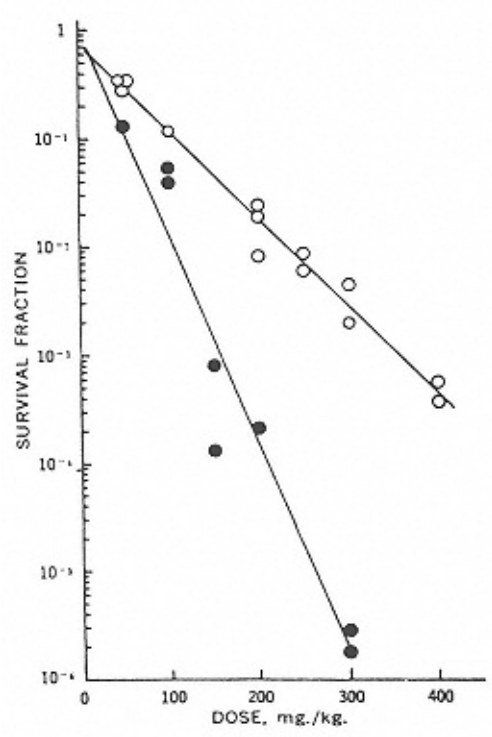

Figure 13
Survival curves for chimera spleen cells (○) and osteosarcoma cells (●) after intraperitoneal administration of single doses of cyclophosphamide to mice. (From Ref. 71.)

the intensity of the pharmacological response can generally not be predicted by simply adding the effects of the two drugs separately and rather complex models may be required. In theory, two types of pharmacodynamic interactions can be identified: competitive and noncompetitive interactions. Competitive interactions occur when two drugs bind to the same receptor. With noncompetitive interactions, each of the interacting drugs binds to a separate receptor; the two drugs can mutually affect either the affinity to the receptor (metaffinoid interaction) or the intrinsic efficacy (metactinoid interaction).

Between the different interaction models important quantitative differences exist. Different equations must consequently be used in their characterization [74,75].

To characterize the competitive pharmacodynamic interaction between two drugs acting at the same receptor, the following equation has been proposed [76]:

$$E = E_0 + \left(\frac{E_{max,1} \cdot (C_1/EC_{50,1})^{n,1} + E_{max,2} \cdot (C_2/EC_{50,2})^{n,2}}{1 + (C_1/EC_{50,1})^{n,1} + (C_2/EC_{50,2})^{n,2}} \right) \quad (20)$$

where E is the pharmacological effect intensity, E0 is the baseline effect value, Emax is the maximal effect, EC50 is the concentration at half-maximal effect, n is a constant expressing the sigmoidicity of the concentration effect relationship, and 1 and 2 are the subscripts referring to the two interacting drugs. If one of the drugs is a competitive antagonist (i.e., Emax,2 is zero), the model can be simplified to [77]:

$$E = E_0 + \left(\frac{E_{max,1} \cdot C_1^{n,1}}{EC_{50,app}^{n,1} + C_1^{n,1}} \right) \quad (21)$$

with:

$$EC50,app = EC50,1(1 + (C2/EC50,2)n,2)1/n,1 \quad (22)$$

This model has been applied to characterize the pharmacodynamic interactions between ligands of the benzodiazepine receptor that differ in intrinsic efficacy [77,78]. In order to characterize the pharmacodynamic interaction between midazolam and flumazenil, rats received 10 or 20 mg of midazolam intravenously in 15 min during a steady-state infusion of flumazenil at rates of 0, 0.25, 0.50, or 1 mg/kg/h. The EEG was continuously monitored and frequent blood samples were collected to determine the pharmacokinetics. Amplitudes in the 11.530 Hz frequency band of the EEG were taken as pharmacodynamic endpoint [12]. The data were subjected to pharmacokinetic-pharmacodynamic modeling to determine the concentration-EEG effect relationship of midazolam [18]. Under the influence of flumazenil a parallel shift in the concentration-EEG effect relationship of midazolam was observed (Fig. 14). The relationship between the flumazenil concentration and the shift in the EC50 of midazolam was nonlinear and could be characterized on basis of Equation (22) yielding the pharmacodynamic parameters of both midazolam (mean ± SE): Emax = 80 ± 5 mV/s, EC50 = 35 ± 3 ng/ml and Hill factor = 1.1 ± 0.2 and flumazenil: EC50 = 24 ± 2 ng/ml and Hill factor = 1.6 ± 0.1. In subsequent studies, rats received various other combinations of midazolam and flumazenil under both steady-state and non-steady-state conditions. These studies demonstrated that the pharmacodynamic parameter estimates of flumazenil are unique and that the competitive interaction model accurately predicts the time course of the EEG effect upon administration of any combination of midazolam and flumazenil [77]. These studies were subsequently extended to the pharmacodynamic interactions between midazolam, the partial benzodiazepine agonist bretazenil, and the inverse agonist Ro 19-4603. Here the model as described in Equation (20) correctly characterized the interactions [78]. The model has been applied successfully in

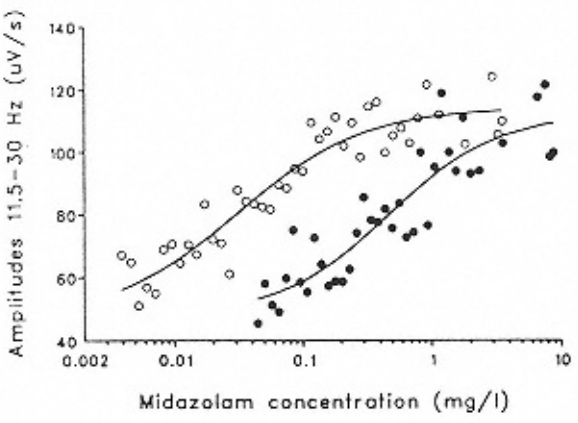

Figure 14
Concentration-EEG effect relationship of midazolam for two rats that had received a continuous infusion of placebo (○)and a steady-state infusion of flumazenil at a rate of 1 mg/kg/h (●). The solid lines represent the best fits according to the sigmoidal Emax model. (From Ref. 77.)

studies on the pharmacodynamics interactions between two drugs in humans [79,80]. In contrast to competitive drug interactions, much less has been accomplished in the area of noncompetitive drug-drug interactions. This remains, therefore, an important area for future research.

The observed effects following administration of a drug may be produced by separate mechanisms via different receptors. A well-known example is the antihypertensive agent clonidine. Following its administration a biphasic response is observed, decrease of blood pressure at low concentration and increase of blood pressure at high concentration [81]. Similar observations have been made for different drugs and pharmacodynamics endpoints [82,83]. A composite pharmacodynamic model has been proposed for this situation [81]. In this model the observed effect is the sum of two opposite effects induced by activity at receptors of two different opposing systems. The relationship between concentration and effect can then be described according to:

$$E = E_0 + \left(\frac{E_{max,1} \cdot C^{n,1}}{EC_{50,1}^{n,1} + C^{n,1}}\right) + \left(\frac{E_{max,2} \cdot C^{n,2}}{EC_{50,2}^{n,2} + C^{n,2}}\right) \quad (23)$$

where the subscripts 1 and 2 refer to the two different effect systems. Although realistic from a pharmacological point of view (for the different drugs, multiple receptor subtypes have indeed been identified), application in vivo is not without difficulties. One of the problems is that both effects may occur at similar concentrations. As a result a high degree of correlation between the various param-

eter estimates is observed and no realistic estimates of the parameters E_{max} and EC_{50} can be obtained. This problem can be solved in part by studying the effects of specific (competitive) antagonists [83,84]. As an alternative, more empirical models may be used to describe the relationship between concentration and effect [51].

D. Tolerance and Sensitization

The phenomenon of decreased effect with prolonged exposure to a drug is called tolerance. Sensitization is the increased sensitivity that may occur upon prolonged treatment with antagonists. Development of both tolerance and sensitization has been recognized for several drugs and can often be explained mechanistically in terms of changes in receptor density, changes in the efficiency of the receptor-stimulus-transfer (i.e., coupling between receptor and second messenger system), depletion of mediators such as catecholamines or physiological adaptation mechanisms. To model the rate and extent of functional tolerance development, modifications of the classic effect model may be used [60]. Such models should ideally allow characterization of both full and partial tolerance as well as rebound and characterization of both full and partial tolerance as well as rebound and characterization of the time course of effect upon continuous as well as repeated intermittent administration [85].

To model the chronotropic and psychological effects of cocaine, pharmacodynamic models have been proposed that model tolerance development as an exponential function acting on the slope S of a postulated linear relationship of cocaine concentration to effect [86,87]. A similar approach has been used to model acute development of tolerance to furosemide-induced diuresis. Here the classic E_{max} model is modified by allowing the parameter EC_{50} to decrease exponentially as a function of the cumulative excretion of furosemide [88]. With these models both full and partial tolerance development can be characterized. A limitation is, however, that the models allow only characterization of the development of tolerance, but not the recovery. As a consequence, they cannot be applied in the situation of repeated intermittent administration of a drug.

One of the more versatile tolerance models presently available has been used to describe tolerance to the effect of nicotine on heart rate (Fig. 15) [85]. In this model, the effect intensity is directly related to drug concentrations in the plasma compartment and the formation of a hypothetical metabolite is postulated that is an antagonist of nicotine. The time course of this metabolite modulates the effect of nicotine. The concentration hypothetical antagonist is assumed to arise by a first-order process, driven by the concentration of nicotine in the central compartment. The model assumes first-order loss of antagonist (tolerance) at a rate k_{ant0}. The parameter k_{ant0} thus determines the rate of appearance and disappearance of tolerance. The inhibitory effect of the metabolite may be

incor-

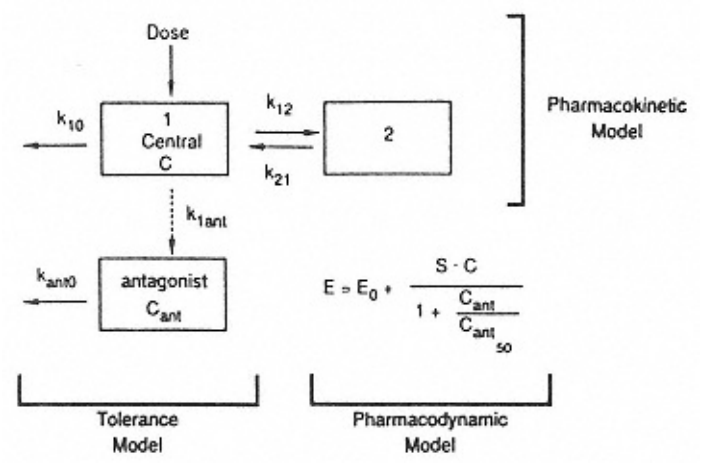

Figure 15
Diagrammatic representation of the pharmacokinetic and pharmacodynamic model proposed for nicotine tolerance. kij, intercompartmental and elimination rate constants; C, concentration of the agonist; Cant, concentration of the hypothetical antagonist; S, slope of the linear relationship between effect and concentration; E, effect intensity; E0, baseline effect intensity. (From Ref. 85.)

porated into the model on the basis of noncompetitive antagonism according to:

$$E = E_0 + \left(\frac{E_{max} \cdot (C/EC_{50})}{(1 + C_{ant}/C_{ant,50})(1 + C/EC_{50})} \right) \quad (24)$$

where Emax and EC50 are the parameters of the Emax model characterizing the concentration-effect relationship of nicotine in the absence of tolerance Cant is the concentration of the hypothetical antagonist expressed in units of steady-state nicotine concentration, and Cant,50 quantifies the degree of tolerance attainable for a given steady-state nicotine concentration. Assuming a linear model for effect in the absence of tolerance development (i.e., nicotine concentrations much lower than the EC50) allows Equation (15) to be simplified to:

$$E = E_0 + \frac{S.C.}{(1 + C_{ant}/C_{ant,50})} \quad (25)$$

where S is equal to the Emax/EC50 ratio of Equation (3) and here is the slope of the linear relationship between nicotine concentration and effect. One of the attractive features of this model is that it allows characterization of both the

development and the disappearance of tolerance (but no rebound). As such it can be applied to study the time course of effect upon repeated intermittent administration (Fig. 16). More recently, the pharmacodynamic model for tolerance to nicotine has been extended to include an effect compartment and to account for arteriovenous concentration differences [89]. This type of modeling has successfully been applied to the pressor effect of caffeine. For this drug the half-life of equilibration of effect with plasma concentration of caffeine was found to be about 20 minutes, whereas the half-life of development and regression of tolerance was about 1 h [90].

A limitation of most models is that they do not allow the characterization of rebound (i.e., a drop of the effect below the original baseline level upon discontinuation of treatment). An empirical model with separate effect and tolerance compartments has been proposed to characterize tolerance and rebound to the antinociceptive effect of morphine [86]. In addition, physiological feedback models have been proposed that may also accommodate rebound [60,92,93]. Such a model has been used successfully to characterize tolerance to the hemodynamic effects of nitroglycerin in experimental heart failure. A key feature of this model is that tolerance development is the result of a counterregulatory effect driven by the extent of the nitroglycerin-induced vasodilation. Thus the pharmacodynamic response rather then the presence of the drug is the driving force in the tolerance development [94].

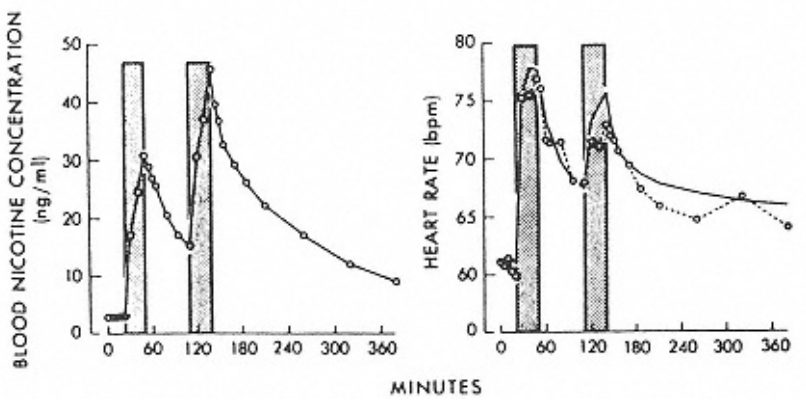

Figure 16
Mean blood concentrations of nicotine (left panel) and the corresponding mean heart rate (right panel) in eight subjects after two 30 min i.v. infusions of 25 mg/kg/min of nicotine, beginning 1 h apart. At the second administration the effect on heart rate is less, despite significantly higher concentrations. The solid line in the right panel shows the fit of the model of Figure 15 to the effect data. (From Ref. 85.)

V. Conclusion

In this chapter various aspects of modeling of the relationship between pharmacokinetics and pharmacodynamics have been reviewed and discussed. Characterization of the relationship between pharmacokinetics and pharmacodynatics requires an integration of realistic pharmacokinetic and pharmacodynamic models. In recent years important progress has been made in this area. For several drugs models are available to characterize and predict the time course of action. Sometimes rather simple direct effect models can be used. In many instances, however, more complex models are required, taking into account the time constants of processes in the pharmacodynamic phase, functional tolerance development, or drug-drug interactions. It is concluded that models of the time course of drug action can be of great value in drug development, since they provide the scientific basis for the dosing regimen in various patient groups.

References

1. J. Dingemanse, M. Danhof, and D. D. Breimer. Pharmacokinetic-pharmacodynamic modelling of CNS drug effects. Pharmacol. Ther. 38:152, 1988.

2. C. A. Shanks. Kinetic-dynamic modelling of neuromuscular blockade. In The In Vivo Study of Drug Action. Edited by C. J. Van Boxtel, N. H. G. Holford, and M. Danhof. Elsevier, Amsterdam, pp. 205217, 1992.

3. H. A. J. Struyker Boudier. Kinetics and dynamics of cardiovascular drug action: Introduction. In The Vivo Study of Drug Action. Edited by C. J. Van Boxtel, N. H. G. Holford, and M. Danhof. Elsevier, Amsterdam, pp. 219231, 1992.

4. P. A. Meredith, H. L. Elliot, and J. L. Reid. An individualized approach to optimizing long-term antihypertensive therapy using concentration-effect analysis. In The In Vivo Study of Drug Action. Edited by C. J. Van Boxtel, N. H. G. Holford, and M. Danhof. Elsevier, Amsterdam, pp. 233252, 1992.

5. C. H. Kleinbloesem, P. Van Brummelen, J. Van Harten, M. Danhof, and D. D. Breimer. Pharmacokinetics and haemodynamic effects of nifedipine in patients with different degrees of impaired renal function. Clin. Pharmacol. Ther. 37:563574, 1985.

6. C. H. Kleinbloesem, P. Van Brummelen, M. Danhof, H. Faber, J. Urquhart, and D. D. Breimer. Rate of increase in plasma concentration of nifedipine as a major determinant of its haemodynamic effects in humans. Clin. Pharmacol. Ther. 41:2630, 1987.

7. A. Wellstein, R. L. Woosley, and S. L. Schwarz. Anti-arrhythmic drugs and cardiac glycosides. In The In Vivo Study of Drug Action. Edited by C. J. Van Boxtel, N. H. G.

Holford, and M. Danhof. Elsevier, Amsterdam, pp. 277302, 1992.

8. J. Dingemanse. Kinetics and dynamics of drug effects on the nervous system: An introduction. In The In Vivo Study of Drug Action. Edited by C. J. Van Boxtel, N. H. G. Holford, and M. Danhof. Elsevier, Amsterdam, pp. 133153, 1992.

9. L. K. Paalzow. Measurement and modelling of analgesic drug effect. In The In Vivo Study of Drug Action. Edited by C. J. Van Boxtel, N. H. G. Holford, and M. Danhof. Elsevier, Amsterdam, pp. 133153, 1992.

10. D. R. Stanski. Pharmacodynamic measurement and modelling of anesthetic depth. In The In Vivo Study of Drug Action. Edited by C. J. Van Boxtel, N. H. G. Holford, and M. Danhof. Elsevier, Amsterdam, pp. 155179, 1992.

11. M. Bührer, P. O. Maître, O. Hung, and D. R. Stansky. Pharmacodynamics of benzodiazepines I: Choosing an EEG parameter to measure CNS effect of midazolam. Clin. Pharmacol. Ther. 48:544554, 1990.

12. J. W. Mandema and M. Danhof. Electroencephalogram measures and relationships between pharmacokinetics and pharmacodynamics of centrally acting drugs. Clin. Pharmacokin. 23:191215, 1992.

13. O. R. Hung, J. R. Varvel, S. L. Shafer, and D. R. Stanski. Thiopental pharmacodynamics. II. Quantitation of clinical and electroencephalographic depth of anesthesia. Anesthesiology 77:237244, 1992.

14. S. L. Shafer. Evaluation quantitative de l'effet morphinique: EEG versus évaluation clinique. In Les Morphiniques. Edited by M. Chauvin. Arnette, Paris, pp. 131146, 1993.

15. R. P. Koopmans, J. Dingemanse, M. Danhof, G. P. M. Horsten, and C. J. Van Boxtel. Pharmacokinetic-pharmacodynamic modelling of midazolam effects on the human central nervous system. Clin. Pharmacol. Ther. 44:1422, 1988.

16. D. J. Greenblatt, B. L. Ehrenberg, J. Gunderman, A. Locniskar, J. M. Scavone, J. S. Harmatz, and R.I. Shader. Pharmacokinetic and electroencephalographic study of intravenous diazepam, midazolam and placebo. Clin. Pharmacol. Ther. 45:356365, 1989.

17. L. T. M. Breimer, P. J. Hennis, A. G. L. Burm, M. Danhof, J. G. Bovill, J. Spierdijk, and A. A. Vletter. Quantification of the EEG effect of midazolam by aperiodic analysis in volunteers. Clin. Pharmacokinet. 18:245253, 1990.

18. J. W. Mandema, E. Tukker and M. Danhof. Pharmacokinetic-pharmacodynamic modelling of the EEG effects of midazolam in individual rats: Influence of rate and route of administration. Br. J. Pharmacol. 102:663668, 1991.

19. J. W. Mandema, L. N. Sansom, M. C. Dios-Vièitez, M. Hollander-Janssen, and M. Danhof. Pharmacokinetic-pharmacodynamic modelling of the EEG effect of benzodiazepines. Correlation with raceptor binding and anticonvulsant activity. J. Pharmacol. Exp. Ther. 257:472478, 1991.

20. J. W. Mandema, M. T. Kuck, and M. Danhof. Differences in intrinsic efficacy of benzodiazepines are reflected in their concentration EEG effect relationship. Br. J. Pharmacol. 105:164170, 1992.

21. M. Danhof, J. W. Mandema, and A. M. Stijnen. Pharmacokinetic complexities of pharmacodynamic studies in vivo. In The In Vivo Study of Drug Action. Edited by C. J. Van Boxtel, N. H. G. Holford, and M. Danhof. Elsevier, Amsterdam, pp. 3160, 1992.

22. M. Danhof and G. Levy. Kinetics of drug action in disease states I. Effect of infusion rate on phenobarbital concentrations in serum, brain and cerebrospinal fluid of normal rats at onset of loss of righting reflex. J. Pharmacol. Exp. Ther. 229:4450, 1984.

23. G. Levy. Correlation between drug concentration and drug response in manpharmacokinetic considerations. In Pharmacology and the Future of Man. Edited by G. H. Achesson. Karger, Basel, pp. 3455, 1973.

24. L. B. Sheiner, D. R. Stanski, S. Voseh. R. D. Miller, and J. Ham. Simultaneous modelling of pharmacokinetics and pharmacodynamics: Application to D-tubocurarine. Clin. Pharmacol. Ther. 25:358371, 1979.

25. M. Danhof, M. Hisaoka, and G. Levy. Kinetics of drug action in disease states II. Effect of experimental renal dysfunction on phenobarbital concentrations at onset of loss of righting reflex. J. Pharmacol. Exp. Ther. 232:435438, 1985.

26. J. W. Mandema, B. Tuk, A. L. Van Steveninck, D. D. Bri-emer, A. F. Cohen, and M. Danhof. Pharmacokinetic-pharmacodynamic modelling of the CNS effects of midazolam and its main metabolite a-hydroxy-midazolam in healthy volunteers. Clin. Pharmacol. Ther. 51:715728, 1992.

27. R. C. Chou and G. Levy. Kinetics of drug action in disease states III. Effect of pregnancy on the relationship between phenytoin concentration and antiseizure activity in rats. J. Pharm. Sci. 73:13481351, 1984.

28. M. Thibonnier, N. H. G. Holford, R. A. Upton, C. D. Blume, and R. L. Williams. Pharmacokinetic-pharmacodynamic analysis of unbound disopyramide directly measured in serial plasma samples in man. J. Pharmacokinet. Biopharm. 12:559574, 1984.

29. W. L. Chiou. The phenomenon and rationale of marked dependence of drug concentration on blood sampling site. Implications in pharmacokinetics, pharmacodynamics, toxicology and therapeuticspart 1. Clin. Pharmacokinet. 17:175199, 1989.

30. W. L. Chiou. The phenomenon and rationale of marked dependence of drug concentration on blood sampling site. Implications in pharmacokinetics, pharmacodynamics, toxicology and therapeuticspart 2. Clin. Pharmacokinet. 17:275290, 1989.

31. D. R. Stanski, R. J. Hudson, T. D. Homer, L. J. Saidman, and E. Meathe. Pharmacodynamic modelling of thiopental anesthesia. J. Pharmacokinet. Biopharm. 12:223240, 1984.

32. A. R. Hansen, K. A. Kennedy, J. J. Ambre, and L. J. Fischer. Gluthetimide poisoning: A metabolite contributes to morbidity and mortality. N. Engl. J. Med. 292:250252, 1975.

33. N. H. G. Holford, P. E. Coates, T. W. Guentert, S. Riegelman, and L. B. Sheiner. The

effect of quinidine and its metabolites on the electrocardiogram and systolic time intervals. Concentration effect relationships. Br. J. Clin. Pharmacol. 11:187195, 1981.

34. C. Crevoisier, W. H. Ziegler, M. Eckert, and P. Heizmann. Relationship between plasma concentration and effect of midazolam after oral and intravenous administration. Br. J. Clin. Pharmacol. 16:51S61S, 1983.

35. J. Dingemanse, D. Thomassen, B. H. Mentink, and M. Danhof. Strategy to assess the role of interactive metabolites in pharmacodynamic studies in vivo: a model study with heptabarbital. J. Pharm. Pharmacol. 40:552557, 1988.

36. M. Danhof, J. Dingemanse and D. D. Breimer. Determination of benzodiazepines in biological fluids. The present day scene. In Bioactive Analytes Including CNS

Drugs, Peptides and Enantiomers. Edited by E. Reid, B. Scales, and I. D. Wilson. Plenum Press, New York, pp. 141148, 1986.

37. D. B. Barnett, and S. R. Nahorski. Drug assays in plasma by radioreceptor techniques. Trends Pharmacol. Sci. 4:407409, 1983.

38. K. Williams, and E. Lee. Importance of drug enantiomers in clinical pharmacology. Drugs 30:333354, 1985.

39. D. E. Drayer. Pharmacodynamic and pharmacokinetic differences between drug enantiomers in humans: An overview. Clin Pharmacol. Ther. 40:125133, 1986.

40. T. Walle, and U. K. Walle. Pharmacokinetic parameters obtained with racemates. TIPS 7:155158, 1986.

41. M. A. Evans, R. L. Nation, L. N. Sansom, F. Bochner, and A. A. Somogyi. Stereoselective drug disposition: Potential for misinterpretation of drug disposition data. Br. J. Clin. Pharmacol. 26:771780.

42. G. T. Tucker, and M. S. Lennard. Enantiomer specific pharmacokinetics. Pharmacol. Ther. 45:309329, 1990.

43. M. Eichelbaum, G. Mikus, and B. Vogelgesang. Pharmacokinetics of (+), (-) and (±) verapamil after intravenous administration. Br. J. Clin. Pharmacol. 17:453458, 1984.

44. B. Vogelgesang, H. Echizen, E. Schmidt, and M. Eichelbaum. Stereoselective first-pass metabolism of highly cleared drugs: studies on the bioavailability of L- and D-verapamil with a stable isotope technique. Br. J. Clin. Pharmacol. 18:733740, 1984.

45. T. Walle, J. G. Webb, E. E. Bagwell, U. K. Walle, H. B. Daniel, and T. E. Gaffney. Stereoselective delivery and actions on beta receptor antagonists. Biochem. Pharmacol. 37:115124, 1988.

46. M. S. Lennard, G. T. Tucker, J. H. Silas, S. Freestone, L. E. Ramsay, and H. F. Woods. Differential stereoselective metabolism of metroprolol in extensive and poor debrisoquin metabolizers. Clin. Pharmacol. Ther. 34:732737, 1983.

47. A. Somogyi, M. Albrecht, G. Kleims, K. Schafer, and M. Eichelbaum. Pharmacokinetics, bioavailability and ECG response oof verapamil in patients with liver cirrhosis. Br. J. Clin. Pharmacol. 12:5160, 1981.

48. M. Eichelbaum. Pharmacokinetic and pharmacodynamic consequences of stereoselective drug metabolism in man. Biochem. Pharmacol. 37:9396, 1988.

49. C. Banfied, R. O'Reilly, E. Chan, and M. Rowland. Phenylbutazone-warfarin interaction in man: further stereochemical and metabolic considerations. Br. J. Clin. Pharmacol. 16:669675, 1983.

50. M. Danhof. Does variability in drug disposition explain (all) variability in drug effects. In Topics in Pharmaceutical Sciences 1989. Edited by D. D. Breimer, D. J. A. Crommelin, and K. K. Midha, FIP, The Hague, pp. 573586, 1989.

51. W. F. Ebling, M. Danhof, and D. R. Stanski. Pharmacodynamic characterization of the electroencephalographic effects of thiopental in rats. J. Pharmacokinet. Biopharm. 19:123143, 1991.

52. W. A. Colburn. Pharmacokinetic/pharmacodynamic modelling: What it is! J. Pharmacokinet. Biopharm. 15:545553, 1987.

53. L. B. Sheiner, D. R. Stanski, S. Voseh, R. D. Miller, and J. Ham. Simultaneous modelling of pharmacokinetics and pharmacodynamics: application to d-tubocurarine. Clin. Pharmacol. Ther. 25:358371, 1979.

54. N. H. G. Holford, and L. B. Sheiner. Understanding the dose-effect relationship: Clinical application of pharmacokinetic-pharmacodynamic modelling. Clin. Pharmacokinet. 6:429453, 1981.

55. E. Fuseau, and L. B. Sheiner. Simultaneous modeling of pharmacokinetics and pharmacodynamics with a nonparametric pharmacodynamic model. Clin. Pharmacol. Ther. 35:733741, 1984.

56. J. Unadkat, F. Bartha, and L. B. Sheiner. Simultaneous modeling of pharmacokinetics and pharmacodynamics with nonparametric kinetic and dynamic models. Clin. Pharmacol. Ther. 40:8693, 1986.

57. P. Veng-Pedersen, J. W. Mandema, and M. Danhof. A system approach to pharmacodynamics III: an algorithm and computer program COLAPS for pharmacodynamic modeling. J. Pharm. Sci. 80:488495, 1991.

58. J. W. Mandema, P. Veng-Pedersen, and M. Danhof. Estimation of amobarbital plasma-effect site equilibrium kinetics. Relevance of poly-exponential conductance functions. J. Pharmacokinet. Biopharm. 19:617634, 1992.

59. P. Veng-Pedersen, and N. B. Modi. Pharmacodynamic system analysis of the biophase level predictor and the transduction function. J Pharm Sci, 81:925-34, 1992.

60. N. H. G. Holford. Parametric models of the time course of drug action. In The In Vivo Study of Drug Action. Edited by C. J. Van Boxtel, N. H. G. Holford, and M. Danhof. Elsevier, Amsterdam, pp. 6169, 1992.

61. D. R. Stanski, J. Ham, R. D. Miller, and L. B. Sheiner. Pharmacokinetics and pharmacodynamics of d-tubocurarine during nitrous oxide-narcotic and halothane anesthesia in man. Anesthesiology 51:235241, 1979.

62. N. H. G. Holford. Clinical pharmacokinetics and pharmacodynamics of warfarin. Understanding the does-effect relationship. Clin. Pharmacokinet. 11:486504, 1986.

63. T. D. Bjorson, and A. Terzic. Pharmacokinetics and pharmacodynamics of anticoagulant drugs. In The In Vivo Study of Drug Action. Edited by C. J. Van Boxtel, N. H. G. Holford, and M. Danhof. Elsevier, Amsterdam, pp. 303311, 1992.

64. M. Pitsiu, E. M. Parker, L. Aarons, and M. Rowland. Population pharmacokinetics and pharmacodynamics of warfarin in healthy young adults. Europ. J. Pharm. Sci. 1:151-57, 1993.

65. C. J. Van Boxtel, and B. Oosterhuis. Pharmacokinetic-pharmacodynamic modelling of

glucocorticosteroid action. In The In Vivo Study of Drug Action. Edited by C. J. Van Boxtel, N. H. G. Holford, and M. Danhof. Elsevier, Amsterdam, pp. 357375, 1992.

66. A. I. Nichols, F. D. Boudinot, and W. J. Jusko. Second generation model for prednisolone pharmacodynamics in the rat. J. Pharmacokinet. Biopharm. 17:209227, 1989.

67. A. I. Nichols, and W. J. Jusko. Receptor mediated prednisolone pharmacodynamics in rats: Model verification using a dose sparing regimen. J. Pharmacokinet. Biopharm. 18:189208, 1989.

68. A. I. Nichols, R. D'Ambrosio, N. A. Pyszczynski, and W. J. Jusko. Pharmacokinetics and pharmacodynamics of prednisolone in obese rats. J. Pharmacol. Exp. Ther. 250:963970, 1989.

69. N. L. Dayneka, V. Garg, and W. J. Jusko. Comparison of four basic models of indirect pharmacodynamic responses. J. Pharmacokinet. Biopharm. 21:457478, 1993.

70. M. J. Moore, and J. J. Theissen. Cytotoxics and irreversible effects. In The In Vivo Study of Drug Action. Edited by C. J. Van Boxtel, N. H. G. Holford, and M. Danhof. Elsevier, Amsterdam, pp. 377400, 1992.

71. W. J. Jusko. Pharmacodynamics and chemotherapeutic effects: Dose-time-response relationship for phase-nonspecific agents. J. Pharm Sci. 60:892895, 1971.

72. W. J. Jusko. Pharmacodynamic model for cell-cycle specific chemotherapeutic agents. J. Pharmacokinet. Biopharm. 1:175182, 1973.

73. C. J. Boxtel, and R. E. Jonkers. Combined drug action. In The In Vivo Study of Drug Action. Edited by C. J. Van Boxtel, N. H. G. Holford, and M. Danhof. Elsevier, Amsterdam, pp. 93111, 1992.

74. B. Oosterhuis, and C. J. Van Boxtel. The kinetics of drug effects in man. Ther. Drug Monit. 10:121132, 1988.

75. E. J. Ariens, and A. M. Simonis. A molecular basis for drug action: the interaction of one or more drugs with different receptors. J. Pharm. Pharmacol. 16:289312, 1964.

76. N. H. G. Holford, and L. B. Sheiner. Pharmacokinetic and pharmacodynamic modeling in vivo. CRC Crit. Rev. Bioeng. 5:273322, 1981.

77. J. W. Mandema, E. Tukker, and M. Danhof. In vivo characterization of the pharmacodynamic interaction of a benzodiazepine agonist and antagonist: midazolam and flumazenil. J. Pharmacol. Exp. Ther. 260:3644, 1992.

78. J. W. Mandema, M. T. Kuck, and M. Danhof. In vivo modelling of the pharmacodynamic interaction between benzodiazepines which differ in intrinsic efficacy. J. Pharmacol. Exp. Ther. 261:5661, 1992.

79. L. T. M. Breimer, A. G. L. Burm, M. Danhof, P. J. Hennis, A. A. Vletter, J. W. H. De Voogt, J. Spierdijk, and J. G. Bovill. Pharmacokinetic-pharmacodynamic modelling of the interaction between flumazenil and midazolam in volunteers by aperiodic EEG analysis. Clin. Pharmacokinet. 20:497508. 1991.

80. R. E. Jonkers, C. J. Van Boxtel, R. P. Koopmans, and B. Oosterhuis. A non steady-state agonist-antagonist interaction model using plasma potassium concentrations to quantify the beta 2 selectivity of beta blockers. J. Pharmacol. Exp. Ther. 249:297302, 1989.

81. L. K. Paalzow, and J. Edlund. Multiple receptor responses: A new concept to describe the relationship between pharmacological effects and pharmacokinetics of a drug: Studies on clonidine in the rat and the cat. J. Pharmacokinet. Biopharm. 7:495510, 1979.

82. L. K. Paalzow, and G. H. M. Paalzow. Concentration-response relations for apopmorphine: effects on heart rate in conscious rats. J. Pharm. Pharmacol. 38:2834, 1986.

83. G. H. M. Paalzow, and L. K. Paalzow. Separate noradrenergic receptors could mediate clonidine induced antinociception. J. Pharmacol. Exp. Ther. 223:795800, 1982.

84. E. Bredberg, and L. K. Paalzow. Effects of apomorphine on heart rate during simultaneous administration of sulpiride: A challenge of the composed concentration-effect model. J. Pharmacol. Exp. Ther. 258:10551060, 1991.

85. H. V. Porchet, N. L. Benowitz, and L. B. Sheiner. Pharmacodynamic model of tolerance: Application to nicotine. J. Pharmacol. Exp. Ther. 244:231236, 1988.

86. M. J. Chow, J. J. Ambre, T. I. Ruo, A. J. Atkinson, D. J. Bowsher, and M. W. Fischman. Kinetics of cocaine distribution, elimination and chronotropic effects. Clin. Pharmacol. Ther. 38:318324, 1985.

87. J. J. Ambre, S. M. Belknap, J. Nelson, T. I. Ruo, S. G. Shin, and A. J. Atkinson. Acute tolerance to nicotine in humans. Clin. Pharmacol. Ther. 44:18, 1988.

88. M. M. Hammarlund, B. Odlind, and L. K. Paalzow. Acute tolerance to furosemide diuresis in humans. Pharmacokinetic-pharmacodynamic modelling. J. Pharmacol. Exp. Ther. 233:447453, 1985.

89. L. B. Sheiner. Clinical pharmacology and the choice between theory and empiricism. Clin. Pharmacol. Ther. 46:605615, 1989.

90. J. Shi, N. Benowitz, C. P. Denaro, and L. B. Sheiner. Pharmacokinetic-pharmacodynamic modeling of caffeine: Tolerance to pressor effects. Clin. Pharmacol. Ther. 53:614, 1993.

91. M. Ekblom, M. Hammarlund-Udenaes, and L. K. Paalzow. Modeling of tolerance development and rebound effect during different intravenous administrations of morphine to rats. J. Pharmacol. Exp. Ther. 266:244252, 1993.

92. E. B. M. Ekblad, and V. Licko. A model eliciting transient responses. Am. J. Physiol. 246:R114R121, 1984.

93. P. Veng-Pedersen, and N. B. Modi. A system approach to pharmacodynamics. Input-effect control system analysis of central nervous system effect of alfentanil. J. Pharm. Sci. 82:266272, 1993.

94. J. A. Bauer, and H. L. Fung. Pharmacodynamic models of nitroglycerin induced hemodynamic tolerance. Pharm Res., 11:816823, 1994.

8
Spatial Imaging of Radioactivity in Animal Tissues and Organs

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I. Introduction.

The distribution of a radiolabeled drug and/or its metabolites in the tissues and organs of a test animal is usually assessed during the course of the pharmacokinetic studies performed prior to the first clinical trials. Up to the early 1980s, the tissue distribution of radioactivity has been determined in most laboratories by dissection of the test animals at room temperature and subsequent processing of the organ and tissue samples by liquid scintillation counting. This method had the disadvantage of allowing postmortem diffusion of radioactive materials to occur in the tissues. Moreover, no information was provided on the specific localization of the radioactive materials in the tissues and organs. In most cases whole-body autoradiography, a spatial radioactivity imaging method that allows for the specific localization of radioactivity in the organs and tissues, was used only to fulfill the requirements of some regulatory authorities for registration of the new drug (i.e., in a very late development phase). One of the reasons for this limited use may have been the difficulty in obtaining accurate quantitative information on a routine basis.

This situation started to change in the late 1980s as new computer-based equipment specifically designed for the qualitative and quantitative interpretation of whole-body autoradiograms, as well as new spatial radioactivity imaging methods, became available. The latter are based either on the generation and recording of photostimulated light (storage phosphor or autoradioluminography) or on direct counting of surface

radioactivity (multiwire proportional counting)

visualized electronically whereas in autoradiography the result is obtained on a photographic emulsion.

This chapter provides the reader with basic information on these spatial radioactivity imaging methods and discusses their strengths and weaknesses in light of their typical applications in drug safety assessment studies.

II. Obtaining Whole-Body Sections

Unlike the dissection/liquid scintillation method, in which *samples* or organs are collected to determine the tissue distribution of radioactivity in the test animal, whole-body animal *sections* are used for all the spatial radioactivity imaging methods. Several sectioning methods were developed in the early years of autoradiography [19]. All have been abandoned due mainly to the limited number of sections of good quality that could be obtained in small laboratory animals or to the difficult working conditions (i.e., the sections were obtained with the help of a sledge microtome in a cold room at -20°C). Nowadays whole-body sections are obtained almost exclusively according to the cryosectioning method of S. Ullberg described in 1977 [1012]. Although many investigators have improved this method slightly, the key steps have not been modified since 1977; first the radioactive dose is administered to the animal. Although many radioisotopes have been already used, most studies are performed with [^{14}C], [^{3}H], or [^{125}I]iodine; the usual dosages are approximately 100 mCi/kg body weight with ^{14}C labeled drugs and 500 mCi/kg with [^{3}H]-labeled drugs; at a preset time point following radioactive dose administration the animal is sacrificed, immediately frozen, and embedded in a precooled medium (i.e., carboxymethylcellulose) to form a solid block; this block is trimmed until the region of interest is reached; 1050 mm thick sections are then obtained by adhesion to a special adhesive tape; selected sections are dehydrated prior to exposure to the film, the imaging plate or into the multiwire proportional counter. For film autoradiography, undehydrated frozen sections may also be exposed. Since the sectioning and section processing steps occur at a low temperature this method guarantees the complete absence of postmortem diffusion of drug-related material in the animal tissues and/or organs.

A. Freezing the Animal

After sacrifice the animal is immediately frozen. Freezing of the animal prior to embedding rather than direct embedding is recommended since the embedding medium acts as an insulator and thus significantly decreases the cooling rate. The temperature of the cooling mixture (usually hexane and solid carbon dioxide) should be stabilized at approximately -80°C to avoid excessive hexane

gas production that also acts as an insulator. N-hexane is preferred over acetone because of the former's poorer solubilizing properties for polar substances at low temperatures; it is thus less able to extract radioactive compounds from the outer skin layers or fur and from the upper respiratory tract. By freezing at a lower temperature (i.e., -170°C) the carcass often splits, mainly in the abdominal region of rodents and eyes. One must take into account that complete freezing lasts at least 1060 min depending on the animal species and size [16]. Thus sufficient time should be allocated for that step. Special attention is also needed during the first minutes of freezing to make sure that the animal is in a stretch position. Ideally the animal is held by its tail and the carcass quenched vertically into the mixture. Some investigators prefer to pour the freezing medium onto the horizontal carcass since a slight compression of the viscera may occur in large animals held vertically. Mice are usually attached to an aluminum plate that helps to prevent twisting of the carcass. After completion of freezing, the excess freezing mixture is rapidly removed from the fur (i.e., with dry tissue wipes). The animal is then embedded or is stored in a deep freezer if embedding is not performed immediately.

B. Embedding

Animal embedding occurs usually in a 2% precooled aqueous gel of sodium carboxymethylcellulose of ultra-low viscosity (Na-CMC). It is convenient to lie the animal on its right side, so that the left side is uppermost in the block and is the first part to be sectioned. This is because the spleen, stomach, and heart lie toward the left side of the body and will be thus sampled while sectioning down to the midline. Moreover it is advised that one remove whenever possible all the hard tissues prior to embedding (i.e., teeth of the large animals: monkey, rabbits, cats) in order to avoid rapid degradation of knives.

In a midsized embedding unit (20 cm × 12 cm) two adult rats weighing approximately 220 g each may be embedded together, allowing thus a decrease in the workload related to sectioning by a factor of 2. To improve the adhesion of small laboratory animals in the embedding medium, the animal fur is removed as rapidly as possible but in less than 23 min with the help of an electrical hair clipper. Excessive heating of the carcass should be avoided. Embedding is performed in four steps. The embedding unit (table + frame) is cooled down to the temperature of the hexane-dry ice freezing mixture; the precooled embedding medium (stored in the refrigerator at +4°C) is then poured slowly into the freezing unit until an embedding basis 1.5 cm in height is achieved. As soon as the latter starts to freeze the carcass is placed into the embedding unit and firmly maintained therein until adhesion is completed. Precooled Na-CMC is then slowly poured over the carcass until it is submerged.

C. Sectioning

In Ullberg's method cryosectioning occurs in a cryomicrotome mounted in a deep-freeze cabinet. The large heavy-duty cryomicrotomes (i.e., the former PMV 2250/450 [S-Broma] the OTSEB Electrolinear Sledge Cryostat (Bright, Huntington, UK) and the new CryoMacrocut [Leica Instruments GmbH, D-6907 Nussloch]) allow sectioning of animals up to 45 cm long (i.e., cat, rabbit, monkey, salmon, trout) [1315] and for subsequent dehydration of the sections in the microtome cabinet. Thus any transfer of the sections prior to complete dehydration and the risk of diffusion of drug-related material due to section transfer are avoided. These cryomicrotomes offer a thoroughly stable electrically driven mobile stage for mounting the specimen and a robust knife held firmly in position between two pillars for cutting without vibration. On the return stroke the knife holder retracts, thus preventing contamination of the block face with debris that may adhere to the underside of the knife. The small cryomicrotomes (i.e., the Leica Polycut [Leica Instruments GmbH, D-6907 Nussloch]) are used only by a limited number of whole-body autoradiography investigators, although these tools allow for sectioning of samples up to 20 cm long (i.e., rodents). Section dehydration, however, occurs in an external freeze-dryer.

To reach the first level of interest (i.e., the level where tissues and organs of interest appear for the first time) the embedding block has to be trimmed. The block should have reached the cryomicrotome temperature before trimming is started. For trimming it is recommended that one use an old knife that may be reserved for that purpose. When the first region of interest is reached, a new knife or a newly resharpened knife is mounted. The characteristics of the knife may differ, especially after resharpening. Therefore single-use blades are preferred and should be used when available (i.e., for the Leica CryoMacrocut). Sections of all the organs and tissues in a rat are obtained at 710 different levels of interest.

To obtain a section, the surface of the block and the edge of the knife are carefully cleaned with the help of a smooth paint brush stored in the cryomicrotome cabinet. The adhesive tape is placed to the top of the block and slightly stuck thereon. All the air bubbles that may have been trapped between the tape and the surface of the block are gently removed by wiping the tape with a clean cold paper tissue. Thereafter a constant pressure is achieved in front of the edge of the knife with the help of a plastic tool whose length is similar to the width of the block while the block is moved. The section adheres to the tape.

D. Section Processing

Except for assessment of the tissue distribution of volatile compounds with film autoradiography, the sections are dehydrated prior to exposure. If dehydration is carried out in the cryomicrotome cabinet, its extent depends not

only on its duration but also on the temperature of dehydration, the number of sections being dehydrated, and their position in the cabinet. This step should be long enough to allow complete dehydration, especially when quantitation if foreseen, since residual water may act as a radiation absorber.

III. Principles of Whole-Body Autoradiography, Autoradioluminography, and Multiwire Proportional Counting/Microchannel Array Detection

A. Whole-Body Autoradiography (QWBAR)

The physical phenomenon on which autoradiography (film autoradiography) is based was discovered in 1867 by Niepce de Saint-Victor. He observed that silver salts were reduced when apposed to urane azotate containing papersheets. He claimed, however, that this persistent activity, similar to that of light, was due to unknown chemical radiations [17]. These facts were confirmed by H. Becquerel in 1896, who noticed that the crystalline lamellas of the double sulfate of uranium and potassium, although light-protected, were able to impress photographic plates when in close contact to the crystalline samples. Becquerel first concluded on a phosphorescence effect [1820] and later on active radiations emitted by the uranium with properties similar to those of the phenomenon observed by Roentgen [21,22].

The process by which an autoradiographic image is obtained is nearly identical to the process by which an ordinary photographic picture is produced and thus depends largely on the processing conditions. Instead of photons, ionizing radiation interacts with the photographic emulsion (i.e., Hyperfilm b-Max, and Hyperfilm-3H [Amersham Plc, Amersham, Bucks, UK] [23]). The photographic emulsion consists of gelatin into which silver halide (usually sliver bromide) crystals are embedded. The films are coated by a protective gelatin layer, except those allowing for the detection of tritium. The latter must therefore be handled carefully to avoid artifacts (fingerprints). If charged particles (such as a or b particles) pass through the emulsion they are liable to interact with silver bromide crystals or gelatin molecules. The interaction of the particles with the crystals in the emulsion is explained by the theory of Gurney and Mott. By ionization, electrons are set free. A negative charge is trapped in the crystal and held first at the sensitivity specks of ionized silver. These specks consist mainly of silver sulfide deposits on the surface of the silver bromide crystals. A latent image is produced; silver bromide crystals have acquired additional electrons and some of the silver ions have become elementary silver atoms. The crystals thus become easily susceptible to reducing agents. The latent image will be converted to the final image (represented by a grain of metallic silver) by the

process of development. Development is carried out manually or automatically in special photographic processors (i.e., Kodak M35) [24]. The nondeveloped crystals are made water-soluble and are removed from the emulsion by the process of fixation [25,26].

B. Autoradioluminography (QWBAL)

The physical phenomenon on which autoradioluminography (also called imaging plate methodology or storage phosphor) is based was discovered by G. Luckey at Kodak in 1975 [27]. The radioactivity-containing samples to be analyzed are exposed to an imaging plate scanned by a laser beam while being converted with high accuracy in a phosphor reader. The imaging plate is a flexible image sensor in which bunches of very small crystals of photostimulable phosphor of barium fluorobromide containing a trace amount of bivalent europium are uniformly coated 150300 mm thick onto a polyester support film. Lattice defects are inherent in the crystal. The grain size of the crystals in approximately 5 mm. Europium acts as a luminescence center. The luminescence mechanism of the BaFBr:Eu^{2+} photostimulable phosphor can be summarized as follows: Part of the Eu^{2+} ions become Eu^{3+} ions through the primary excitation by ionizing radiations, with electrons being released into the conduction band. These electrons are trapped into the Br-ion empty lattices of the lattice defects of the crystal, and color centers of the metastable state are formed. When the photostimulated excitation light (PSL) to be absorbed by the color center is irradiated, the trapped electrons are liberated again into the conduction band. The bluishpurple PSL released upon laser excitation is collected through the light collector guide to the photomultiplier tube and converted there to analog electric signals in chronological order and subsequently to digital signals [2832]. After scanning in the phosphor reader, all the data recorded on the imaging plate can be quickly erased by exposing the imaging plate to uniform light in an imaging late eraser attached. The imaging plate can then be reused almost indefinitely.

The most widely used phosphor-imagers are the Fuji BAS 1000, BAS 2000, and BAS 3000 (Fuji Photo film Co. Ltd., Tokyo, Japan) and the phosphorImager 425E and SF (Molecular Dynamics, Sunnyvale, CA), and, to a lesser extent, the GS-250 Molecular Imager (Bio-Rad Labs, Hercules, CA).

C. Multiwire Proportional Counting/Microchannel Array Detection (MWPC/MICAD)

Multiwire proportional counting (MWPC) is a direct radioactivity counting method, which is best known through Prof. Charpak's Nobel Prize winning technology: the ionizing particle leaves a trail of ions as it passes through a chamber filled with a mixture of argon and methane. These ions generate pulse changes in the multiwire system that picks up the charge signals at their position

of origin. A decoding system identifies this position and increments the contents of the corresponding computer memory position. Thus multiwire proportional counters (i.e., the Ambis 4000 [Radioanalytic System], and the Digital Autoradiograph [Berthold, D-7547 Wildbad]), may be regarded as sophisticated new generation counters allowing for real-time analysis. However, to avoid contamination of the multiwire system, these counters are fitted with a protective sheet (i.e., Mylar foil), rendering the quantitative detection of weak b-particles such as tritium impossible. Because of the relatively poor optical resolution achieved in the past with the early multiwire proportional counters, many attempts have been made in recent years by the MWPC manufacturers to develop new detectors offering a better optical resolution. Presently the best detector is the Microchannel Array Detector (MICAD) present in the InstantImager [Packard Instrument Co, Meriden, CT], developed by A. Jeavons. The MICAD offers a resolution compatible with most of the general applications of these imaging techniques (see below) [3335].

IV. Evaluation of Spatial Radioactivity Imaging Results.

Compared to the dissection-liquid scintillation counting method, the spatial imaging techniques offer many important advantages thanks to the guarantee of the absence of diffusion of radioactivity in the sample to analyze and to the good resolution achieved by these methods. Whole-body autoradiography, autoradioluminography, and multiwire proportional counting allow a very precise localization of radioactivity in the organs and tissues independent of their size, location, and characteristics. The specific distribution of radioactivity in the different tissues and organs is thus assessed even in the organs contaminated by a biological fluid. These methods also offer adequate archiving. The results are documented as an image and a file, in contrast to the results in liquid scintillation counting, which are in the form of files only. For example, in the wholebody [^{14}C]autoradiogram presented in Figures 1 and 2, the concentration of radioactivity observed in the renal cortex is different from that observed in the renal medulla and that in the splenic red pulp differs from that in the splenic white pulp. Therefore, results in terms of a mean concentration value for the kidney and the spleen make little sense. In Figure 3, radioactivity is concentrated in very small tissue cartilage.

V. Quantitation of Radioactivity in Whole-Body Sections

A. Choice of Standards

Because of the number of parameters influencing more or less significantly the efficiency in autoradiography, autoradioluminography, and multiwire propor-

tional counting, quantitation of the radioactivity in the region of interest occurs necessarily by comparison to the radioactivity concentration of a reference material. Until the 1980s typical materials used as reference were either a film dipped into a radioactive solution [3640] or a sheet of methacrylate, polymethacrylate, or Perspex of a known concentration of radioactivity [4147]. However, the influence of the self-absorption of the material taken as a standard or a reference compared to that of the tissue to be analyzed, as well as of the section characteristics (i.e., thickness and dehydration conditions) has been neglected or insufficiently addressed [4852]. For example, it was assumed that the infinite thickness of a section for b-particles emitted by ^{14}C was 30 mm. Moreover, a single reference material 12 mm thick was used for the quantitation of radioactivity in sections whose thickness was as different as 30, 40, 50, or 60 mm.

The self-absorption in [^{14}C]autoradiography has been demonstrated to be similar in all major organs in the rat [53] but to be very different from that of nonbiological material (i.e., polymethacrylate). In [^{14}C] radiograms of 40 mm thick sections of untreated rats obtained after exposure of these sections between a homogeneous radioactive ^{14}C source and a photographic emulsion the optical density values in the different tissues were not significantly different from those in blood, except in bone mineral and adipose tissue (higher self-absorption) and vitreous body (lower self-absorption). The high density of bone mineral and very low density of the vitreous body explained these results, whereas the high self-absorption in the adipose tissue, unexpected based on the low-density of this tissue was an artefact due to the incomplete dehydration of this tissue. This has been proven after reexposure of the sections used for assessing tissue self-absorption following intensive dehydration.

Since radiograms similar to those described above cannot be obtained with [^{3}H]-labeled compounds because of the low energy of the particles [42,54]. We must assume that the self-absorption of weak b-particles is also nearly the same in all the organs.

B. Importance of Normalization of Working Conditions

The influence of section characteristics (mainly their thickness) on the final result has been clearly shown [53,55]: the optical density values do not reach a plateau when one is exposing sections up to 80 mm thick, suggesting that for ^{14}C autoradiography using dehydrated tissue sections a thickness of 80 mm may still not be considered as infinite. It is suggested that one check carefully the thickness of the samples and standard sections used for quantitation [56]. Adipose tissue is actually dehydrated more slowly than the other tissues [57]. In conclusion, it is essential to work under normalized conditions in order to estimate accurately the concentration of radioactivity in tissue sections. Therefore, and

Figure 1
14C Whole-body autoradiogram shows the distribution of a 14C-labeled drug and/or its radioactive metabolites in the tissues and organs of a male rat. The black areas correspond to the high radioactivity concentrations.

At 0.5 h following a single 20 mg/kg subcutaneous dose (200 mCi/kg body weight) the animal was sacrificed by an intravenous injection of isoflurane, immediately frozen in a mixture of n-hexane and dry ice at -75°C, and embedded in a 2% precooled gel of sodium carboxymethylcellulose; 40 mm thick sagittal sections were obtained according to the method of S. Ullberg in a PMV 2250/450 cryomicrotome (LKB Instr., S-Bromma) at -23°C. Selected sections were dehydrated for 60 h in the cryomicrotome at -20°C and then mounted onto 2 mm thick aluminum plates and exposed for 15 days at -23°C onto Hyperfilm b-max (Amersham Plc, Amersham, UK). After exposure the films were developed automatically in a modified M35 Kodak photographic processor. The autoradiograms were evaluated qualitatively and quantitatively with the help of a MCID/OS2 image analyzer (Imaging Research, Saint Catherine's, Ontario, Canada). For quantitation a polynomial calibration curve was established with the intensity values of the photographic impressions obtained from a radioactive blood scale processed under

the same conditions as the samples to analyze. The highest radioactivity concentrations are observed in the cartilage, renal cortex, and
liver, amounting to approximately 24, 22 and 9 mg-equivalents of parent drug/g tissue, respectively.

because of the specific self-absorption of dehydrated tissues, a radioactive whole-blood scale obtained under the same conditions as the tissue and organ samples to be analyzed may be used as a reference material, assuming that the labeled drug added to the blood aliquots is distributed equally in the different blood components.

C. Calibration Curves

For calibration we recommend a radioactive blood scale obtained by dilution of a rat radioactive blood sample of a known concentration of radioactivity with rat fresh blood. Depending on the detection method selected (film, imaging plate, or MWPC/MICAD), linear or nonlinear calibration curves have to be used. The equation of the calibration curves depends on the analysis method and, to a lesser extent, on the total range of concentrations covered. Since the response curve of a photographic emulsion is nonlinear [36], nonlinear calibration curves are needed to quantitate film autoradiograms. The number of concentration data points should be sufficient to define accurately the parameters of the polynomial function used to fit the calibration curve. The minimal and maximal concentrations have to be in the range of the expected tissue concentrations. For example, radioactivity concentrations of 0.4 to 2500 dpm/mg are adequate when working with the usual ^{14}C radioactive doses. A factor of one to 10 should be applied when working with ^{3}H-labeled compounds. These ranges allow one to determine

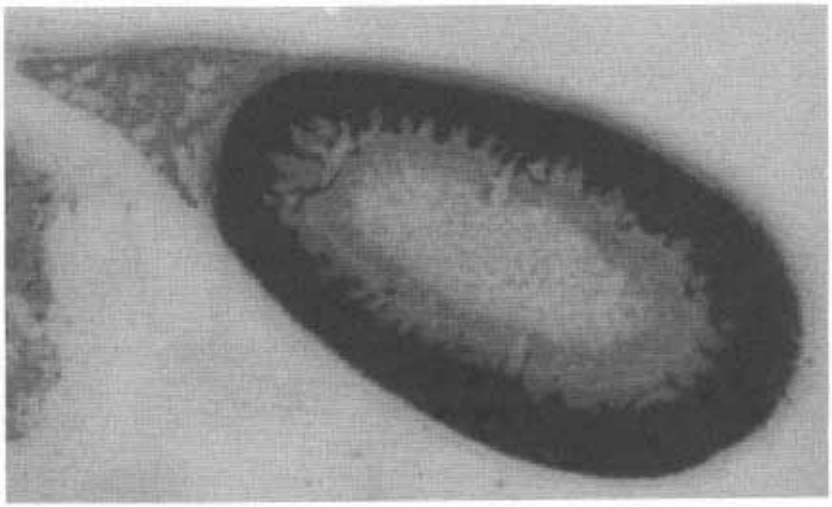

Figure 2
Selected area of a 14C whole-body autoradiogram showing the distribution of a 14C-labeled drug and/or its radioactive metabolites in the spleen and kidney of a male rat. The black areas correspond to the high radioactivity concentrations. Method was as in Figure 1. At 72 h the radioactivity concentration in the renal cortex, renal medulla and pelvis amount to approximately 41, 22, and 2 mg-equivalents of parent drug/g tissue, respectively. In the splenic red pulp and splenic white pulp the concentrations amount to 11 and 3 mg-equivalents of parent drug/g tissue, respectively. Calculation of mean concentration values in the kidney and spleen therefore makes little sense.

accurately the limit of determination (the lowest absorbance value significantly different from the background value) and for which concentration saturation of the film occurs. The limit of detection (the lowest concentration detected) and limit of saturation thus depend on the experimental conditions (i.e., duration of exposure, counting efficiency, background value and the characteristics of the equipment, and for autoradiography only, film processing). The response of phosphor imagers and multiwire proportional counters is theoretically linear over a wide range of concentrations, and quantitation using the phosphor imagers available is presently based on this assumption.

In autoradiography, calculation of the calibration curves and sample analysis are performed using an image analyzer [58,59]. Among the analyzers the MCID/OS2 (Imaging Research, St. Catherine's, Ontario, Canada) appears to be most appropriate. For autoradioluminography and multiwire proportional counting the analyzer included in the equipments may be used. However, because of their

Figure 3
Selected area of the 14C whole-body autoradiogram presented in Figure 1 shows the distribution of a 14C-labeled drug and/or its radioactive metabolites in the bones of a male rat. The black areas correspond to the high radioactivity concentrations (for the experimental conditions, see Fig. 1).
The radioactivity concentrations in the cartilage and in the bone marrow amount to 24 and 1.4 mg-equivalents of parent drug/g tissue, respectively, whereas apparently the bone mineral is free of radioactivity (limit of detection: 5 ng-Eq/g). Because of the very small size of cartilage in the rat it would not have been possible to investigate the specific distribution of drug-related material in the bone with a nonspatial radioactivity imaging method (i.e., liquid scintillation counting).

actual limitations we prefer an external image analyzer (i.e., MCID/OS2), assuming that both pieces of equipment are compatible.

D. Limits of Detection and Quantitation

To compare the limit of detection and quantitation achieved with the spatial imaging methods to that of liquid scintillation counting one has to differentiate between small and large samples. In liquid scintillation counting the limit of detection is related to the total amount of radioactivity in the vial and consequently of the weight of the sample, whereas in the spatial imaging methods it is independent of the sample size. For whole-body autoradiography (QWBAR), autoradioluminography (QWBAL), and MWPC, the limit of detec-

tion is taken as the lowest absorbance, PSL, or net counts value obtained from a radioactive blood scale significantly higher than the measured blank value (one sided t-test, $p = 0.05$).

In the case of a ^{14}C label the limit of detection using QWBAR may be as low as 0.4 dpm/mg, assuming an 11 week exposure duration and long-term autoradiographic background reduction [60]. With QWBAL and MWPC, concentrations as low as 2 dpm/mg may be detected within less than 3 days of exposure. This means that for large samples (50 mg and more) liquid scintillation counting may help to detect lower concentrations than spatial imaging methods (0.2 dpm/mg are achieved under optimal conditions). In contrast, for small samples (weighing less than approximately 5 mg), the limit of detection using liquid scintillation counting is higher (i.e., 510 dpm/mg). Thus spatial radioactivity imaging methods allow the detection of lower ^{14}C concentrations in small tissues.

In the case of ^{3}H-labeled compounds a concentration of 40 dpm/mg can be routinely detected by autoradiography after an 11 week exposure, whereas the limit of detection using liquid scintillation counting amounts to approximately 1.5 and 10 dpm/mg for typical 50 mg and 5 mg samples, respectively. Using autoradioluminography we detected a concentration of 150 dpm/mg of tissue after a 3 day exposure. This clearly demonstrates that the limit of tritium detection achieved with spatial imaging methods is not as low as with liquid scintillation counting.

VI. Comparison of Whole-Body Autoradiography, Autoradioluminography, and Multiwire Proportional Counting/Microchannel Array Detection

A. QWBAR

Among the three methods QWBAR offers the best optical resolution. This allows accurate qualitative results even in tissues as tiny as the ocular membranes, the blood vessel wall, the intestinal and gastric mucosa, the renal medullocortical junction, and some specific cerebral regions. Autoradiography allows also the detection of the presence of a mottled pattern that may characterize the distribution of a drug in a tissue (i.e., the liver).

Moreover, since nondehydrated sections may be used because films may be exposed at a low temperature as easily as a room temperature, QWBAR allows to localize selectively volatile and nonvolatile metabolites in a single whole-body section using selective subsequent exposure.

QWBAR also offers the best absolute limit of detection, assuming long exposure periods are acceptable. For instance, concentrations as low as 0.4 dpm/

mg of tissue may be detected on a routine basis after an 11 week exposure onto Hyperfilm b-Max films. In addition QWBAR provides the investigator with an original document (film) that can easily be archived and be presented in meetings (as an overhead transparency).

On the other hand the photographic process is rather difficult to standardize. Although one may avoid variations in the photographic process by using specially developed automatic film processors, it is strongly suggested that one expose the calibration standards together with the samples to analyze each film for accurate quantitation. This is, of course, film-consuming and time-consuming since for each film a new calibration curve has to be generated. Moreover, expensive chemicals are used and wasted, which may turn out to be an environmental issue.

In addition the dynamic range is very narrow compared to that of QWBAL and MWPC/MICAD, although sufficient in most cases following a ^{14}C radioactive dose of approximately 100 mCi/kg; for example, 1 dpm/mg as well as 2000 dpm/mg of tissue are routinely detected following a 7 week exposure, assuming the films are adequately processed. However, for tissues with a lower or higher radioactivity concentration, the sections are reexposed for a longer or shorter period, respectively.

The accuracy of quantitation depends on the concentration due to the nonlinear response of the photographic emulsion. It is lower for the concentrations for which the slope of the calibration curve tends to be more or less parallel to the concentration axis (polynomials of the 4th or 5th degree are used to plot the optical density values versus the radioactivity concentration). The low coefficient of variation of 10% reported previously held true only for radioactivity concentrations between 100 and 1400 dpm/mg of tissue [55]. In addition, several standards containing low and high concentrations of radioactivity are necessary to determine accurately the limit of determination and the maximal concentration which may be estimated.

Despite its strengths QWBAR belongs unfortunately already to the past and will most likely disappear progressively. This becomes even more evident when one considers the low number and poorer performance of the new emulsions developed in the last years. The film manufacturers are no longer interested in developing new photographic emulsions because of the silver price and/or new processing materials.

B. QWBAL

Autoradioluminography represents a good compromise between the three spatial radioactivity imaging methods. The new large phosphor imagers (those allowing exposure of whole-body rodent sections) offer a resolution of approximately 160 mm, which is not

as good as that obtained in film autoradiography

but allows one to cover almost all the applications of QWBAR, except the assessment of mottled patterns in a tissue. The resolution achieved with this equipment may, however, be improved in the future; in fact it has been shown to be much better in smaller phosphor imagers with a smaller scanning step. However, the total number of data obtained in the latter per unit of area is so high that much larger computer capacities are needed. Thus it is presently not realistic to apply this technology to the phosphor imagers used for large animals whole-body sections.

In addition autoradioluminography offers a wide dynamic range over 5 orders of magnitude; this range may be even wider if the performance of the photomultipliers would be as good as that of the imaging plates. Moreover, the response of the phosphor imager is almost linear over the entire dynamic range. Thus quantitation in autoradioluminography is easy and necessitates a limited number of internal standards. However, using an external calculator it has been shown that for low concentrations (i.e., less than 20 dpm/mg) the equation of the calibration curve differs significantly from that obtained for higher concentrations [61]. The reason for this deviation has not been clearly identified. It appears that it might be related to the way the background value is subtracted from the sample value. Thus, the estimation of radioactivity concentrations below 20 dpm/mg of tissue may be inaccurate if one does not take this limitation into account.

Another strength of QWBAL is its speed: the short exposure time needed to detect radioactivity in a sample. Compared to film autoradiography, a speed factor of approximately 1040 is achieved, which is more realistic than the factor of 100 claimed in the commercials. This gain may be of great practical importance since it allows one to decrease the exposure period of a typical sample from 7 weeks to less than 2 days (see below).

On the other hand, the electronic images generated cannot be handled as films, being visualized only on the display monitor. Making overheads, hard copies, and/or slides for discussion (i.e., in a meeting room) or including the images in a document results in a lower apparent quality. Moreover, since the electronic images are not displayed in a 1:1 scale to the sections, direct super-position of the image onto the corresponding section for better identification of the region of interest and interpretation is only possible through size-correction of the electronic image.

With respect to the higher speed, this does not mean better sensitivity, and the minimal amount of radioactivity detectable by QWBAL is not as small as that with QWBAR. For instance a radioactivity concentration as low as 0.4 dpm/mg of tissue is not detectable by QWBAL. This is due mainly to the fact that in autoradioluminography an exposure duration of over 4 days does not allow one to achieve a significantly better detection limit,

compared to 2 or 3 days, because of the increase of the background value, even when using special shield

boxes. Moreover, higher speed does not necessarily mean that the total duration of a study is shorter. This depends on the total number of samples to analyze, the number of imaging plates available, and the time needed for analysis. For example, we obtained with a ^{14}C-labeled drug administered intravenously at a radioactive dose level of 200 mCi/kg b.w. similar qualitative and quantitative results with QWBAR and QWBAL after an exposure period of 7 weeks and 2.5 days, respectively. Thus, for a limited number of samples (maximum 16 whole-body rat sections, assuming the investigator owns four imaging plates, exposes four sections per plate, and is able to analyze these plates within one working day), the difference is very significant (i.e., 7.2 weeks vs. 3.5 days). In contrast, for a large number of samples (for a complete distribution study in male and female rats at five or six time points following oral and parenteral administration a minimum of 400 sections is exposed) the time difference becomes much smaller. Of course, for QWBAR the duration of the exposure remains unchanged since the 100 films will be exposed for 7 weeks and processed simultaneously at the end of this exposure period. For autoradioluminography, assuming the investigator owns 10 plates, he or she will have to use 10 times each plate, reading it, erasing it, and checking prior to reexposure that it has been correctly erased, which may be time-consuming. A maximum of 20 imaging-plates can be exposed a week. The total duration of the exposure is thus 5 weeks at minimum, which does not differ much from the 7 weeks necessary when using QWBAR. However part of the information is obtained within a few days, whereas for autoradiography one has to wait until the exposure is completed.

Another issue is the tailing effect we have observed for radioactivity concentrations higher than 2000 dpm/mg. It may be due to the relatively low performance of the photomultipliers, which saturate rapidly due to the high scanning speed of the plate (a 20 cm × 24 cm imaging plate is scanned in approximately 3 min). This tailing effect is a limitation for accurate quantitation in the numerous regions of interest localized next to areas where such radio-activity concentrations may exist (i.e., the stomach and intestinal content, urine, bile and tissues showing a particular affinity to drug-related material). If a tailing effect is observed, reexposure for a shorter duration is mandatory.

The phosphor imagers offer inadequate image analysis software packages. This limitation is, however, circumvented when the detector is connected to a high-quality analyzer (i.e., MCID/OS2). Fuji has already commercialized the BAS/MCID imager, which is a combination of the Fuji BAS 2000 and the MCID/OS2 analyzer. Molecular Dynamics also is in the process of improving the analytical performance of their equipment.

Detecting weak b-emitters (i.e., tritium) may still be regarded as partial limitation of phosphor imagers, although it is theoretically possible with all the phosphor imagers since

they are all based on the same technology. For the BAS imagers Fuji Photo commercializes special imaging plates specifically designed

for that purpose. However, these plates are intended primarily for limited use only and may be reused at most for three to four exposures, which may turn out to become very time-expensive.

Detecting volatile metabolites may also be an issue in QWBAL, since the nonhydrated sections have to be placed onto the imaging plate, exposed, and removed from the plate at an adequate low temperature. Thus it is extremely difficult to avoid humidity due to condensation being trapped onto the surface of the plate, which will lead to the generation of artifacts and rapid degradation of the plate.

Another limitation of QWBAL is the risk of definitive contamination of the imaging plate by radioactive compounds being absorbed into the sensitive layer, leading to hot spots that cannot be erased. This explains why many investigators place a thin plastic foil between the sample and the imaging plate, decreasing, however, the sensitivity, speed, and resolution of the system.

C. MWPC/MICAD

MWPC is the most accurate method in terms of quantitative results and offers the highest speed, the widest dynamic range, a full linearity over the whole range of concentrations, a real-time analysis, and the well-established technology of the proportional direct counting. The response of the multiwire proportional counter is fully linear and completely proportional to the exposure duration, thus allowing an accurate calibration curve with a limited number of standards. It can be estimated that the speed factor is 310 and the dynamic range is wider by a factor of 10 compared to autoradioluminography. The very wide dynamic range may, however, not be relevant for usual tissue distribution studies. Even more than QWBAL, the speed may be relevant only in the case of a limited number of samples (see below) since only one sample (i.e., four to five whole-body rat sections) can be exposed simultaneously. This single exposure leads to a total exposure period, in the case of a high number of samples, that is even longer than with film autoradiography. However, this issue may be minimized by developing multiplate racks for automatic subsequent exposure of several samples.

Real-time analysis is convenient since it allows visualization of the electronic image on the display monitor and termination of the exposure when desired. Unlike film and storage phosphor screens the multiwire proportional counter response, when normalized to the instrument counting time, is independent of acquisition time [62]. Thus, since the counting efficiency has been shown to be constant for different tissues, data can be easily converted to dpm results without the routine inclusion of internal standards. Moreover, since the ^{14}C counts per minute are independent of the counting time, it is not always

necessary to wait until an image of high quality is generated, assuming the region of interest may

be defined with accuracy. Also results obtained after different exposure durations may be compared directly without having to expose standards with each sample. For example, radioactivity concentrations estimated after 3 days of exposure with QWBAR were estimated accurately already after 20 min of exposure in the InstantImager (the new MWPC by Canberra Packard) because we *knew* where to analyze (using the section and the previous results). In contrast, an 18 h exposure would have been necessary to generate a picture from an unknown sample with a contrast similar to that obtained by QWBAR and QWBAL.

Multiwire proportional counting and microchannel array detection have however some limitations. Because of the high risk of contamination (the sample being entered directly into the counter), a Mylar foil has to be placed on top of the sample prior to exposure, rendering detection of tritium impossible. In addition, since the samples are loaded *into* the counter, only dehydrated sections can be used, thus excluding the detection of volatile compounds that may be lost through dehydration and/or by processing at room temperature.

The resolution, although adequate for most typical applications of spatial radioactivity imaging techniques (except when 3H is used) is not as good as for the two other techniques. For instance, it allows one to get information on the distribution of radioactivity in the major tissues but is not sufficient to assess accurately the distribution of drug-related material in very small tissues and organs nor to check whether a specific targeting occurs in some tissues (i.e., mottled pattern in liver).

VII. Typical Applications of WBAR, WBAL, and MWPC in Drug Metabolism Studies.

Typical applications of spatial imaging techniques in animal pharmacokinetics in particular and drug safety assessment in general are primarily all those related specifically to the important and precise local information obtained with these techniques, thanks to the fact that images are obtained with good resolution. In addition these methods are more than useful as alternatives to the conventional tissue distribution methods. The major applications of these methods in drug safety assessment are related to their good resolution: distribution as well as retention and/or accumulation of radioactive material in small tissues (splenic white and red pulp, uveal tract, blood vessel wall, cartilage, specific cerebral and cerebellar regions, skin layers), distribution/retention/accumulation of drug-related material within a single organ (renal cortex, renal medulla, corticomedullar junction and, and pelvis), and specific distribution pattern of radioactivity within a single tissue (i.e., mottled pattern in the liver).

As for the dissection/liquid scintillation counting method, one of the most typical

applications is the support of regular toxicology and toxicokinetic studies

by investigating the tissue distribution as a function of time (part of the ADME program) in the same animal species under the same conditions as those of the toxicology studies [6370]. The target tissues and target organs are detected and an overall estimation of the organ and tissue exposure is gained. By assessing the placental transfer and the distribution of radioactive material in the embryos and fetuses, the fetal exposure to the drug and/or its metabolites is assessed, supporting the teratology studies [7174].

In addition, thanks to the high speed of phosphor imaging and multiwire proportional counting, specific applications may be envisaged for these techniques in exploratory studies (i.e., targeting) when a limited number of samples is sufficient for interpretation of the results [7578]. For instance, these techniques may help to validate some tests (i.e., the micronucleus test) by demonstrating that bone marrow of the mouse is exposed to drug-related material.

Spatial radioactivity imaging methods also help to support the animal pharmacokinetic studies. At the end of the collection period they allow one to demonstrate clearly whether or not drug-related material is retained in the carcass since the residual radioactivity may not be detected due to the high factor of dilution existing when doing carcass radioactivity analysis. However, as stated above, these applications are not realistic for multiwire proportional counting when a tritium-labeled compound is administered and for WBAL and MWPC/MICAD when nondehydrated sections are required.

Film autoradiography should therefore be used specifically when an optimal resolution is required to assess, for example, the affinity of melanin to the ocular membranes [7984]; when a large number of samples have to be analyzed (distribution study as a part of the animal pharmacokinetic program); when very low tissue radioactivity concentrations are expected; and for documentation. Multiwire proportional counting and microchannel array detection may be preferred in preliminary studies when almost instantaneous estimates of the radioactivity concentrations in the tissues should be obtained to design the main studies and when very accurate quantitative results are required. As an alternative, autoradioluminography represents a good compromise among these methods and may therefore be used for almost all these applications. One must be aware that for a large number of samples, the total duration of the study may not be significantly shorter than with autoradiography.

VIII. Conclusion

Classic film autoradiography (QWBAR), although offering the best resolution, the largest field of application, and the best detection limit, will disappear progressively and will be replaced by MWPC/MICAD and QWBAL. The latter may be considered as the method of

choice for tissue distribution studies, since among the spatial imaging methods they represent a good compromise between

speed, quality, and performance. Multiwire proportional counting and microchannel array detection should be considered mainly for specific applications and accurate quantitation. Dramatic improvements are envisaged in the near future in the fields of QWBAL (imaging plate of high quality and less easily contaminated, high software performance, better archiving, full acceptability) and MWPC (higher sensitivity and better optical resolution), whereas the prices of all equipment might drop.

References

1. S. Ullberg. Studies on the distribution and fate of ^{35}S-labeled benzylpenicillin in the body. Acta Radiol. (Stockh) Suppl. 118:1110, 1954.

2. S. Ullberg et al. Distribution of radioactive iron in pregnant mice studies by whole-body autoradiography. Acta Radiol. (Stockh) 55:145155, 1961.

3. S. Ullberg et al. Autoradiographic distribution studies with natural oestrogens. Acta Endocrinol. (Kbh) 43:7586, 1963.

4. Y. Cohen et al. Modification de la méthode d'autoradiographie de S. Ullberg sur coupes de souris entières. C.R. Soc. Biol. (Paris) 153:300304, 1959.

5. P. Pellerin et al. Low-temperature autoradiography for the detection of tritium in tissue, with removal of luminescence induced by tritium. Nature 184:13851386, 1959.

6. P. Pellerin. La technique d'autoradiographie anatomique à la température de l'azote liquide. Pathol. Biol. (Paris) 9:233252, 1961.

7. L. E. Martin et al. A simple low temperature radioautographic technique. Biochem. J. 82:1718, 1962.

8. P. Chiadot et al. Etude autoradiographique des tissus et des excrétats du rat après administration de D.T.P.A.-^{59}Fe(III). Pathol. Biol. (Paris) 12:10191084, 1964.

9. F. Kalberer. A new method of macroautoradiography. In Advances in Tracer Methodology 3. Plenum Press, New York, 1966, pp. 139147.

10. S. Ullberg. The technique of whole-body autoradiography. Cryosectionning of large specimens. Science Tools 229, 1977.

11. S. Ullberg et al. Autoradiography in pharmacology. In Radionuclides in Pharmacology 1. IEPT, 1978, pp. 221239.

12. S. Ullberg et al. Localization in relation to action. In Radioisotopes in Pharmacology. Edited by Waser and Glasson. 1979.

13. P. Bénard et al. Etude autoradiographique de la distribution du tritium chez des singes macaques traités par un extrait de *Ruscus* tritié. Ann. Pharm. Franç 43(6):573584, 1985.

14. T. Hay et al. The disposition of ivermectin in Atlantic salmon *(Salmo salar).* Pharmacol. Toxicol. 67:307312, 1990.

15. W. J. Waddell et al. The disposition of [14C]acrylamide in rainbow trout studied by whole-body autoradiography. Fundam. Appl. Toxicol. 14:8487, 1990.

16. C. G. Curtis et al. Preparation of Animals for Autoradiography. In Whole-Body Autoradiography. Academic Press. 1981, pp. 1731.

17. A. Niepce de Saint-Victor. Sur une nouvelle action de la lumière. C.R. Acad. Sc. (Paris) 65:505507, 1867.

18. H. Becquerel. Sur les radiations émises par phosphorescence. C.R. Acad. Sci. (Paris) 122:420421, 1896.

19. H. Becquerel. Sur les radiations invisibles émises par les corps phosphorescents. C.R. Acad. Sci. (Paris) 122:501503, 1896.

20. H. Becquerel. Sur quelques propriétés nouvelles des radiations invisibles émises par divers corps phosphorescents. C.R. Acad. Sci. (Paris) 122:559564, 1896.

21. H. Becquerel. Sur les radiations invisibles émises par les sels d'uranium. C.R. Acad. Sci. (Paris) 122:689694, 1896.

22. H. Becquerel. Emission de radiations nouvelles par l'uranium métallique. C.R. Acad. Sci. (Paris) 122:10861088, 1896.

23. E. Ehn et al. Properties of an antiscratch-layer-free X-ray film for the autoradiographic registration of tritium. Sci. Tools 26(2):2429, 1979.

24. A. Schweitzer. Automatic processing of Hyperfilm b-Max. A.R.N. 1:1416, 1988.

25. L. E. Feinendegen. Autoradiography: the photographic image. In 3H-Labeled Molecules in Biology and Medicine, 4. 1967, pp. 167175.

26. T. H. James et al. Reduction of silver halides and the mechanism of photographic development. Chem. Rev. 30:132, 1942.

27. G. Luckey. US patent 3, 859, 527, 1975.

28. T. Hamaoka. Autoradiography of new era replacing traditional x-ray film. Cell Technol. 9:456462, 1990.

29. R. F. Johnson et al. Autoradiography using storage phosphor technology. Electrophoresis 11:355360, 1990.

30. J. Miyahara: Visualizing things never seen before. The imaging plate: A new radiation image sensor. Chem. Today, 2936, 1989.

31. S. Shionoya. Mechanistic approach toward photostimulated luminography. Radioluminography 1(1):115, 1992.

32. A. Shigematsu. Bao-Bei, or the powerful technology, in science of whole-body metabolism. Autoradioluminography. Radioluminography 1(3):115, 1992.

33. A. P. Jeavons. Method and apparatus for quantitative autoradiography analysis. US patent 5,138,168, 1992.

34. A. P. Jeavons et al. The high density avalanche chamber for positron emission tomography. IEEE Trans. Nucl. Sci. 30:640645, 1983.

35. D. Englert et al. Microchannel array detector for quantitative electronic radioautography. Cell. Mol. Biol.

36. S. A. M. Cross et al. A quantitative method for measuring radioactivity in tissues sectioned for whole-body autoradiography. Int. J. Appl. Radiat. Isot. 25:381386, 1974.

37. S. A. M. Cross. A quantitative method for whole-body autoradiography. Acta Pharmacol. Toxicol. 41, Suppl. 1:3233, 1977.

38. D. T. Masuaka et al. An in vitro brain autoradiographic technique and a digital microdensitometer for the quantitation of autoradio-grams. Acta Pharmacol. Toxicol. 41, Suppl. 1:5455, 1977.

39. M. Berlin et al. Accumulation and retention of mercury in the mouse. Arch. Environ. Health 6:589601, 1963.

40. B. J. Scott et al. A comparison of 12 X-ray films for the autoradiography of isotopes emitting weak b-particles. Int. J. Appl. Radiat. Isot. 34(4):765767, 1983.

41. G. M. Alexander et al. Quantitative measurement of local cerebral metabolic rate of glucose utilizing tritiated 2-deoxyglucose. Brain Res. 223:5967, 1981.

42. G. J. Falk et al. Radioautographic efficiency for tritium as a function of section thickness. Radiat. Res. 20:466470, 1980.

43. E. R. Franklin. Factors affecting the sensitivity of x-ray films used for whole-body autoradiography. Xenobiotica 13(3):163169, 1983.

44. R. D. Irons et al. Standardization and calibration of whole-body autoradiography for routine semiquantitative analysis of the distribution of ^{14}C-labeled compounds in animal tissues. Toxicol. Appl. Pharmacol. 59:250256, 1981.

45. S. Longsaw et al. A poly (methyl-^{14}C)methacrylate source for use in whole-body autoradiography and beta-radiography. Xenobiotica 8:289295, 1978.

46. S. Longsaw et al. The development of a permanent, robust, stable, standard radioactive source for use in whole-body autoradiography. Acta Pharmacol. Toxicol. 41 (Suppl.):52, 1977.

47. H. Kutzim. Die quantitative Bestimmung der Verteilung von S^{35}-Sulfat bei der Maus mittels Autoradiographie. Nuklearmedizin 3:3950, 1963.

48. E. R. Franklin. The response of selected X-ray films to ^{14}C-b-radiation. Int. J. Appl. Radiat. Isot. 31:256258, 1980.

49. R. W. Hendler. Self-absorption correction for carbon 14. Science 130:772777, 1959.

50. B. Larsson et al. A film for rapid registration of tritium. Sci. Tools 3032, 1977.

51. A. W. Rogers. The analysis of autoradiographs. In Techniques of Autoradiography. Elsevier Amsterdam, 1973, 329354.

52. H. R. Schutte. Absorption und Selbstabsorption. In Radioaktive Isotope in der Organischen Chemie und Biochemie. Verlag Chemie, 1966, pp. 2324.

53. A. Schweitzer. Autoradiographie qualitative et quantitative. Application à une substance dopaminergique (CU 32085) et à l'un de ses métabolites principaux (CH 29717) chez le rat. Thesis, University of Nancy, France, 1984.

54. A. M. Kellerer et al. Criteria of applicability for autoradiography of tritium. Environment. Biophys. 183191, 1993.

55. A. Schweitzer et al. A simple method for the quantitation of [14C]whole-body autoradiograms. Int. J. Appl. Radiat. Isot. 38(5):329333, 1987.

56. T. Ito et al. Variation in thickness of the large cryosections cut for whole-body autoradiography. Int. J. Appl. Radiat. Isot. 42(2):187192, 1991.

57. A. Schweitzer, unpublished results.

58. C. Goochee et al. Application of computer-assisted image processing to autoradiographic methods for studying brain functions. TINS: 256260, 1983.

59. M. Masseroli et al. Automatic quantitative evaluation of autoradio-graphic band films by computerized image analysis. Life Sci. 59(20):331336, 1993.

60. R. K. Traub et al. Quantitative assessment and reduction of long-term autoradiographic background. J. Histochem. Cytochem. 38(4):581583, 1990.

61. A. Schweitzer, unpublished results.

62. A. Schweitzer and D. Englert. Rapid quantitation of 14C in whole-body cryosections. In Application Note for the InstantImager, 1994.

63. P. Bénard et al. Application of whole-body autoradiography in toxicology. CRC 15(2):181215.

64. E. R. Franklin et al. Whole-body autoradiography. In Xenobiotic Metabolism and Disposition: The Design of Studies on Novel Compounds. Edited by H.P.A. Illing. CRC Press, Boca Raton, 1989, pp. 4166.

65. H. K. Bhat et al. Toxicokinetics of monochloroacetic acid: A whole-body autoradiography study. Toxicology 63:3543, 1990.

66. A. G. E. Wilson et al. Application of whole-body autoradiography in toxicology. Toxicol. Methods 3:147160, 1992.

67. M. Lemaire et al. Disposition of Sandostatin, a new synthetic somatostatin analogue, in rats. Drug Metab. Dispos. 17(6):699703, 1989.

68. W. McNally et al. Quantitative whole-body autoradiographic determination of tacrine tissue distribution in rats following intravenous or oral dose. Pharm. Res. 6(11):924930, 1989.

69. U. Busch. Whole-body autoradiography (WBAR): use for pilot studies of pharmacokinetics in rats. Br. J. Clin. Toxicol. 41, Suppl. 1:2829, 1977.

70. A. E. Ahmed et al. Whole-body autoradiographic disposition, elimination and placental transport of [14C]tri-o-cresyl phosphate in mice. J. Appl. Toxicol. 13(4):259267, 1993.

71. P. Benard et al. Transplacental passage and milk excretion of radioactivity in laboratory animals dosed with 14CF 2207: A quantitative whole-body autoradiography study. In Synthesis and Applications of Isotopically labelled Compounds. Proceedings of the Third International Symposium, Innsbruck, Austria, July 1721, 1988.

72. S. Ullberg et al. Placental passage and fetal accumulation of labelled vitamin B12 in the mouse. Arch. Int. Pharmacodyn. Ther. 167:431449, 1967.

73. S. Ullberg. Autoradiography in fetal pharmacology. In Fetal Pharmacology Symposium, Stockholm. Edited by L. Boreus. Raven Press, New York, 1971, pp. 5573.

74. W. J. Waddel. Localization and metabolism of drugs in the fetus. Fed. Proc. 31:52, 1972.

75. G. Akel et al. Distribution and tumor penetration properties of a radiosensitizer 2-[14C] misonidazole (Ro 07-0582) in mice and rats as studied by whole-body autoradiography. Cancer Chemother. Pharmacol. 17:121126, 1986.

76. I. Fand et al. Use of whole-body autoradiography in cancer targeting with radiolabeled antibodies. Cancer Res. (suppl.) 50:885s891s, 1990.

77. F. Laurent et al. Autoradiographic distribution of [^{14}C]-labelled pimonidazole in rhabdomyosarcoma-bearing rats and pigmented mice. Cancer Chemother. Pharmacol. 22:308315, 1988.

78. T. Tokui et al. Contribution of serum proteins as carriers of antitumor agent RS-1541 (Palmitoyl Rhizoxin) in mice. Biopharm. Drug Dispos. 15:93108, 1994.

79. P. Larsson et al. Disposition of ^{3}H-Aflatoxin B$_1$ in mice: Formation and retention of tissue bound metabolites in nasal glands. Pharmacol. Toxicol. 67:162171, 1990.

80. P. A. Zane et al. Physicochemical factors associated with binding and retention of compounds in ocular melanin of rats: correlations using data from whole-body autoradiography and molecular modeling for multiple linear regression analysis. Pharm. Res. 7(9):935941, 1990.

81. R. H. Liss et al. In Advances in Modern Toxicology: New Concepts in Safety Evaluation 1. Edited by Mehlman, Shapiro, and Blumenthal. Hemisphere, Washington, DC, 1976, pp. 273305.

82. H. Ghantous et al. Accumulation and turn-over of metabolites of toluene and xylene in nasal mucosa and olfactory bulb in the mouse. Pharmacol. Toxicol. 66:8792, 1990.

83. G. B. Cassano et al. Autoradiographic distribution in mice with ^{14}C-imipramine. Int. J. Neuropsychiatry 2:269, 1966.

84. A. Schweitzer. Spatial imaging of radioactivity: application in drug safety assessment. Proceedings of the 5th international symposium on the synthesis and applications of isotopes and isotropically labelled compounds, Strasbourg, France, 1994.

9
Recent Developments in Drug Metabolism Methodology

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I. Introduction

The focus of metabolism research in drug discovery/development has been greatly influenced by advances made in molecular biology and bioanalytical methods. Integration and application of these new and/or improved technologies to drug metabolism are providing researchers the ability to characterize the metabolic fate of drugs not only at the intact organism, tissue, organ, or cellular levels but also at a molecular level. Identification and characterization of drug-metabolizing enzymes have enhanced our ability to predict and understand drug-drug interactions, factors influencing drug disposition such as pharmacogenics and environment, species differences in metabolism, and reactive metabolite chemistry.

The importance of characterizing the metabolic fate of drugs at the molecular level was recently highlighted in an editorial by Food and Drug Administration (FDA) officials in which a potentially life-threatening interaction was described between the antihistamine terfenadine and the antifungal agent ketoconazole [1].

The high costs and risks associated with drug development have resulted in increased utilization of metabolite screening technologies to drug discovery/design efforts. Pharmacophore modeling of cytochromes P450 and applications of heterologous expressed cytochromes P450 are areas of research that are offering promise in predictive human metabolism [25].

Our intention in this chapter is to highlight some of the recent advances in metabolism research. The characteristics of these advances lend themselves to categorization into one of three areas: in vitro and in vivo methodologies, and bioanalytical detection and identification techniques.

II. In Vitro Methodologies

The primary in vitro model systems used in drug metabolism involve hepatic enzymes and preparations from animal and human origin. Several extrahepatic sites of metabolism such as intestine and brain can, however, be used [6,7]. The focus of this section will be on in vitro methodologies employing hepatic enzymes and tissue. Advances in molecular biology and enzymology have allowed for the identification and characterization of over 200 individual cytochromes P450 genes [8]. A detailed description of the cytochromes P450 nomenclature based on amino acid sequence alignment and divergent evolutionary relationships is provided by Nelson et al. [8]. For the purposes of human drug metabolism, the primary cytochromes P450 subfamilies of interest include CYP1A, CYP2C, CYP2D, CYP2E, CYP3A, and CYP4A. Depending on the specific metabolic

issues being examined, a variety of in vitro model systems differing in biological complexity and flexibility are available. In this section, an effort is made to proceed from the isolated enzyme to subcellular fractions, to whole cell, tissue slices, and, finally, whole organ systems.

A. Enzyme Systems

Single or isolated enzyme systems are a powerful technique for the study of enzymatic processes under controlled conditions in which substrate, enzyme, and cofactor concentrations can be easily maintained and manipulated. The single enzyme system is very useful in the study of enzyme kinetics, specificity, and mechanisms [9,10].

The classic approach to obtaining isolated drug-metabolizing enzymes was to extract, isolate, purify, and reconstitute the enzyme of interest from the appropriate animal or human tissue [1113]. Advances in molecular biological methods now provide an alternative approach. Specific cDNA sequences for a particular drug-metabolizing enzyme can be cloned and heterologously expressed in an appropriate prokaryotic or eukaryotic (preferably mammalian) cell line [5]. The first successful cDNA mediated cytochrome P450 expression was accomplished in 1985 by Oeda et al. with the expression of P450MC (CYP1A1) in *Saccharomyces cerevisiae* [14]. Since then, many forms of drug-metabolizing enzymes have been expressed, including the cytochromes P450 [15,16], flavincontaining monooxygenases [17], glucuronyltransferases [18], sulfotransferases [19], epoxide hydrolases [20], glutathione S-transferases [21], and N-acetyltransferases [22]. Expression of these enzymes is generally performed in stable cell lines that have little or no endogenous activity for the enzyme being expressed. A more detailed discussion of enzyme expression systems can be found in Chapter 10. Expressed enzymes can be studied in a physiological environment (e.g., intact cells) or in subcellular fractions (e.g., microsomes). These expression systems are particularly useful in evaluating the potential role of individual enzymes in drug metabolism processes and also in predicting and interpreting drug-drug interactions [5]. Moreover, large quantities of expressed enzymes can be used in an applied biocatalysis manner to produce new pharmaceuticals or metabolites [4].

As powerful as this technique is, a word of caution should be added in evaluating the results from single or isolated enzyme systems. Correlations between other in vitro techniques and in vivo data should be made to ensure accuracy of results, specifically, heterologous expressed enzymes in which factors such as folding or posttranslational modifications of the protein, presence or absence of required cofactors and/or enzymes, and expressed enzyme activity may differ from the native state [5,23].

B. Subcellular Fractions

Xenobiotic metabolism occurs within cytoplasmic and/or organelle sites throughout the cell. Subcellular fractions, separated by cell disruption and differential centrifugation, are a simplified system compared to the whole cell or tissue preparations for the investigation

of selective metabolism issues [24,25].

The isolation of these fractions results in preparations that are concentrated in the enzyme(s) of interest, such as cytochromes P450 and glucuronyltransferases in microsomes, or b-oxidation enzymes in the case of mitochondria or peroxisomes. Another advantage of subcellular fractions is their long-term stability when stored at -80°C. This makes them particularly useful as convenient and accessible preparations.

Microsomes are the most frequently employed subcellular fraction, and the dominant in vitro model system. This subcellular component, composed of endoplasmic reticulum, contains most of the oxidative drug-metabolizing enzymes, such as the cytochromes P450 and flavin monooxygenases, glucuronyltransferases, epoxide hydrolases, alcohol dehydrogenases, esterases, and methyltransferases [26]. There are many examples throughout the literature of the use of microsomal preparations. For example, Berthou et al. compared the species differences and quantitative importance of cytochromes P450 1A1 and 1A2 in the metabolism of caffeine with liver microsomes obtained from humans, monkey, rabbit, rat, and mouse [27]. Mani et al. illustrated how liver microsomes can be manipulated by means of induction and inhibition to differentiate between the cytochrome P450-mediated and flavin monooxygenase-mediated metabolism of tamoxifen [28,29]. Identification of the CYP3A enzyme in the human microsomal metabolism of the HMG-CoA reductase inhibitor lovastatin was made by using antibodies specific for CYP3A [30]. Microsomes have also been employed in conjunction with target cells such as human lymphocytes for studying metabolic activation and subsequent cytotoxicity processes [31,32]. The ability to phenotype microsomes, especially of human hepatic origin, can greatly increase the utility of this model system in assessing the involvement of specific cytochromes P450. For example, CYP2E1 catalytic activity was found to correlate with defluorination of the anesthetic agent enflurane [33].

Another useful subcellular component, the cytosolic fraction (100,000 × g supernatant), contains the sulfotransferases, glutathione S-transferases, epoxide hydrolases, alcohol dehydrogenases, dihydrodiol dehydrogenase, catechol O-methyltransferases, and N-acetyltransferases [26]. More specialized organelle components, such as mitochondria and peroxisomes, contain the enzymes involved in boxidation of both endogenous and exogenous compounds. Mitochondrial fractions were used to study valproate b-oxidation [34]. Moreover, mitochondria contain several forms of cytochrome P450 involved primarily in steroid biochemistry [35].

C. Cellular Systems

Cell culture or cell suspensions offer a sophisticated opportunity to study many aspects of both drug metabolism and toxicology within a physiological environment that contains normal concentrations of enzymes and cofactors, yet can be

manipulated under appropriate conditions. In similarity to in vivo circumstances, all xenobiotics must compete with endogenous metabolic pathways for biotransformation and drug/metabolite transport in and out of the cell. These systems can be used to evaluate multiple aspects of drug metabolism, drug transport across cell membranes, enzyme induction, and cytotoxicity from such organs as kidney, intestinal mucosa, and liver. Cells can be derived from primary or permanent cell cultures. Experiments involving primary cell lines are more predominant in drug metabolism studies, because permanent cell lines possess little or none of the enzymes involved in xenobiotic metabolism [36]. The following discussion of cellular systems will be limited to the description of hepatocytes as they are commonly applied to drug metabolism studies.

The most common means of isolating hepatocytes is by the two-step collagenase perfusion method [37,38]. Once isolated, the cells can either be used immediately in cell suspension experiments or placed in primary culture for longer-term experiments. One disadvantage of primary culture experiments is the rapid decline in levels of cytochromes P450 with time [36]. The stabilization of cytochromes P450 in primary cultures is an area of intense research with several new methods of culturing, such as changes in media composition or coculturing with other cell types, that lead to greater stability of the enzymes [36,39]. For drug metabolism studies, we prefer to use isolated cell suspensions because of the native levels of drug-metabolizing enzymes present immediately after isolation. Human hepatocytes are an exception to this general method because they tend to improve in viability and metabolic profiles when placed in culture.

It is important to mention that human hepatocytes are becoming more prevalent as an in vitro system because of the greater availability of fresh human tissue from various institutions and commercial sources. For example, cultured human hepatocytes were shown to be a useful tool in predicting drug-drug interactions [40]. Their use should provide superior comparisons of in vitro and in vivo human metabolism of xenobiotics than are possible with animal cell cultures [41]. Developments in cryopreservation methodology have improved the viability and drug-metabolizing capability of stored hepatocytes from animal and human tissue [4244]. The ability to store isolated cells is a distinct advantage because of the laborious nature of isolating cells and the random access to fresh human liver [45,46].

D. Liver Slices

In the past, organ slices were extensively used to study a variety of biochemical processes. Their use slowly disappeared because of the technical requirements of producing viable slices of equal thickness, and the improvement of cell culturing techniques. Today, the ability to produce precision cut organ slices has

become much easier with the availability of commercial tissue slicers [47,48], and improved organ (tissue slice) culture conditions [49]. Organ slices have been isolated from many different species including humans [50], and from several organ types, primarily liver, brain, heart, and kidney [51]. Liver slices offer some advantages to the previously mentioned in vitro systems. For example, slices maintain a higher level of cellular organization with cell-cell junctions remaining intact. Moreover, the slices are not exposed to proteolytic enzymes (e.g., collagenase) that may strip cells of important membrane receptors necessary for biological function. In comparison to other in vitro methods, organ slices can be more easily and rapidly produced, with some experiments running for longer periods of time. Several experiments have been performed to evaluate the potential differences between liver slices, hepatocytes, and liver microsomes [52,53]. These studies suggest little difference in the cytochrome P450-mediated metabolism of such compounds as caffeine between the various in vitro systems.

Initial experiments using liver slices promoted their use for the study of toxicology [54]. For example, liver slices were used as a method to examine hepatotoxic potential of acetaminophen [55]. Today, liver slices are showing increased usage in drug metabolism studies. For example, liver and kidney slices from human, dog, and rat were used to examine the biotransformation of an ergot derivative, CQA 206291 [52] and tolbutamide [56]. Besides species comparisons, this technique can be used to predict intrinsic clearance values as shown with CQA 206291 [52]. Whereas liver slices contain both phase I and phase II drug-metabolizing enzymes, Gunawardhana et al. employed human liver slices to study the N-acetylation of sulfamethazine [57].

E. Organ Perfusion.

The perfused organ method is an excellent opportunity to measure toxicological and pharmacokinetic events and parameters because of its close approximation to the in vivo tissue environment. The technique, its applications to the study of endogenous biochemical processes, and the effects/mechanisms of toxins and xenobiotics have been described in detail [58,59]. Organ perfusion offers several advantages over other in vitro methods: preservation of organ architecture (vasculature, bile canaliculi, cellular junctions, and zonal distribution of enzymes within the organ); the ability to regulate perfusate flow rate; and two sampling sites available for determination of substrate and metabolite concentrations (perfusate and bile). A limitation of organ perfusion experiments, however, is that only one experiment can be performed per animal. This is in contrast to other in vitro methods that allow for multiple experiments from an individual animal preparation. This limitation, therefore, makes liver perfusion experiments more applicable to drug development projects as opposed to drug discovery

programs, in which lower numbers of preparations are generally needed.

The usefulness of this technique for studying pharmacokinetics and drug metabolism-related issues is primarily focused on the prediction of in vivo pharmacokinetic parameters, such as extraction ratio, intrinsic clearance, and first pass metabolism. Some examples of the use of perfused rat liver include the effect of paracetamol on liver function, the effect of dose size on the disposition of nifurtimox, the clearances of carcinogenic nitrosamines, and the drug-drug interaction of quinine and quinidine [6063]. More directly related to drug metabolism was the study of cyclohexanecarboxylate in the perfused rat liver [59,64]. This study confirmed the liver as the major organ involved in cyclohexanecarboxylate metabolism. Several minor metabolites were identified as not being formed in the liver, indicating extrahepatic metabolism. These minor metabolites were subsequently found to be formed in isolated kidney tubules.

F. Regulation and Characterization of Metabolic Pathways

Many of the in vitro preparations discussed above are well suited for the study of biochemical toxicology, cytotoxicity, irreversible drug-protein binding, drug metabolism, and enzyme regulation (e.g., induction and inhibition). Most relevant to this discussion is the induction and inhibition of drug-metabolizing enzymes. For more detailed information on the topic of enzyme induction and inhibition, the reader is directed to other reviews [6567]. Induction of drug-metabolizing enzymes can have a dramatic impact on the disposition, toxicology, and metabolic profile of the agent under study or other drugs or xenobiotics taken concomitantly. Enzyme induction in animals can be assessed usually by one of two ways: the animals can be induced in vivo followed by organ removal or in vivo animal dosing with probe substrates; or cells or slices can be prepared from normal animal tissues and treated in culture to induce enzymatic activity in vitro [68]. Induction studies with human tissues are ethically difficult unless organs can be obtained postmortem that were naturally induced in vivo (e.g., smoking to induce 1A or alcoholism to induce 2E1). Another approach is to culture human hepatocytes or slices with the agent suspected of induction or inhibition [69]. For example, Pichard et al. used cultured human hepatocytes to screen a series of 59 xenobiotics for their potential to induce or inhibit CYP3A [70].

Elucidation and characterization of enzymes involved in a metabolic pathway can be very useful in predicting adverse interactions, as well as interpreting known drug-drug interactions. Several methods are available to determine the enzymes involved in the metabolism of xenobiotics, including specific inducers, chemical inhibitors, immunoinhibition of enzymes, correlation of substrate metabolism and cytochrome P450 enzyme activity, and incubation with single enzyme systems. Several methods, in combination, are generally employed to positively identify the enzyme(s) involved

[71,72]. Some reported chemical

inhibitors include gestodene as an inactivator of human CYP3A4 [73], furafyl-line as an effective inhibitor of human CYP 1A2 [74], and ethacrynic acid as a potent inhibitor of glutathione S-transferases [75]. Investigators should be aware that some inhibitors and inducers are species- and isoenzyme-specific and may not be useful under all circumstances [74,75]. Characterization of enzymes by determination of kinetic parameters, such as K_m (Michaelis-Menton constant), V_{max} (maximum velocity), and K_i (inhibition constant) can be very useful in choosing appropriate substrate concentrations for in vitro studies (K_m and V_{max}), predicting in vivo pharmacokinetic parameters (V_{max}/K_m to determine intrinsic clearance), and in measuring or ranking the potency of drug-drug interactions (K_i).

The preceding discussion is an overview of the properties and manipulative powers of several in vitro systems as applied to drug metabolism and pharmacokinetic studies. The choice of in vitro method and its manipulation is ultimately defined by the question under investigation. Whenever possible, several methods should be used along with in vivo confirmation of the results. In the future, as more information is accumulated from these in vitro studies, a database of results and comparisons with in vivo data will allow researchers to place more confidence in in vitro results as predictors of in vivo drug metabolism and pharmacokinetic parameters and phenomenon.

III. In Vivo Methodologies

The challenges in conducting in vivo metabolic disposition studies are greater today than at any other time. For example, the need to develop highly potent and specific drugs along with the development of peptidomimetic agents and oligonucleotides from the field of biotechnology necessitate development of improved drug and metabolite separation, profiling, and detection methodologies. There is now the realization that both pharmacogenetic and environmental factors influence metabolism, which has necessitated the need to quantify those influences. Moreover, there is a definite need to validate accurately the metabolic capabilities of animal species used in toxicology studies. In addition, animal usage issues have also placed constraints on in vivo metabolism studies [76]. A successful drug development program will require a carefully thought out in vivo metabolism component.

Although in vitro metabolism studies can provide information on enzymology and structure-function relationships, in vivo studies are required to examine the additional factors contributing to a compound's overall metabolism such as absorption, distribution, extrahepatic metabolism, first-pass effect, protein binding, and disease state. The primary biological fluids used to identify and characterize the metabolic fate of a drug have been urine, bile, feces, and, to a lesser extent, seminal fluid, breast milk, and saliva. The

examination of these excretion prod-

ucts, however, often does not provide insightful information on the underlying metabolic pathways or on the nature and amounts of drug-derived metabolites present in the systemic circulation.

In vivo metabolism studies are generally classified into preclinical and clinical studies. The focus of these investigations is to assess the routes of excretion, extent of metabolism, and the amounts of metabolites formed, circulating, and being excreted. These types of studies are labor- and time-intensive, requiring the development of specific and sensitive analytical methods. Because of the intensive nature of these studies, they are seldom performed during the early discovery stage of drug evaluation except to address such issues as bioavailability and elimination half-life. They are more commonly planned and conducted once a drug candidate is chosen for development towards an investigational new drug application (IND). Although, technically, only minimal drug metabolism information is required for an IND submission, it often is in the interest of the sponsor to conduct more extensive studies to anticipate potential clinical development issues.

A. Radionuclides

The availability of a radiotracer tag on a drug candidate greatly simplifies the ability to monitor the formation and excretion of metabolites. The position selected for placement of the radiotracer tag is ideally at a chemically and metabolically stable site. Thus, any radiolabeled material detected during chromatographic separation of the matrix is assumed to be drug-derived [77,78]. The two most common radionuclides used as tracer tags in drug metabolism studies are tritium (^{3}H) and carbon 14 (^{14}C). Tritiated compounds can be synthesized with relatively high specific activities; however, difficulties with isotope effects and exchange with endogenous protons can confound metabolism studies. Carbon 14 cannot be synthesized with the same high specific activity of a tritiated analogue; however, isotope effects and the potential of exchange are not usually an issue. Thus, ^{14}C is generally the nuclide of choice for in vivo metabolism studies.

It is anticipated that with increasing pressure on the disposal of radioactive waste the use of radionuclides in drug disposition studies will diminish. Alternative approaches, such as stable isotope labels with mass spectrometric detection, may see greater usage. At present, few studies have used stable isotope labeling and, thus, only limited information is available to assess the accuracy of this methodology compared to radionuclides [79].

B. Animals

Preclinical drug metabolism studies are generally conducted in the same animal species

selected for in the pharmacological and/or toxicological development

program. Initial in vivo studies often focus on drug bioavailability and pharmacokinetic issues. With these studies, analyses of plasma and/or urine extracts may show the presence of an unidentified component. To simplify metabolite identification and quantitation, radiolabeled drug is generally used, although nonlabeled or stable labeled analogues may also be used [80].

Initial drug disposition studies commonly focus on mass balance or recovery of a radioactive dose. Results generated are used to correlate routes of excretion and metabolism with pharmacokinetics. Urine and feces are typically collected for nonspecific (total radioactivity) and specific (profiling of parent and/or metabolites) analyses, although collection of bile, blood, plasma, brain, selected tissues, and expired CO_2 may be required. Differences between nonspecific measurements of total radioactivity and specific quantification of parent drug are suggestive of the presence of metabolites.

In the event that mass balance (typically > 90%) is not attained within the species, protocols may be altered to prolong the collection period. As an alternative, radioactive analysis of expired CO_2 and the entire animal carcass may be necessary to achieve mass balance. Moreover, analysis of the animal carcass may be helpful in identifying tissues acting as drug compartments or sites of drug equivalent sequestration. Although not described here, regulatory guidelines in other countries (Japan) may require higher accountability of the administered radioactive dose.

1. Bile Duct Cannulation

Significant levels of radioactivity observed in feces following oral administration suggest either lack of drug absorption or biliary excretion. To address the issue of lack of absorption, feces can be extracted and profiled. The role of biliary excretion can be examined in bile duct cannulation studies in which bile, urine, and feces are collected and profiled [81].

2. Regulation

Pretreatment of animals with inducing agents such as phenobarbital (CYP2B) [82,83], b-naphthoflavone (CYP1A) [83], clofibrate (CYP4A) [84], and ethanol (CYP2E1) [85] can alter the metabolic capacity of animals and allows examination of the role of the respective cytochromes P450 in in vivo animal metabolism. Moreover, gender [86], fasting [87], and disease state (e.g., streptozotocin-induced diabetic rat) [88] can be examined under in vivo conditions. The influence of glutathione status, the major intracellular nonprotein thiol, on detoxification processes, such as with acetaminophen, can also be examined [89].

During multiple dose pharmacology and/or toxicology studies, the potential for induction of metabolic enzymes should or may need to be assessed depending on such variables as compound class (i.e., anticonvulsants), plasma concen-

trations of parent, and increased or decreased tissue or animal weight. If induction or inhibition of in vivo metabolism is suspected, the liver is typically removed and its metabolic capacity assessed by methods similar to those used in vitro. Species differences in regulation should also be investigated [90].

3. Whole Body Autoradiography

Quantitative whole body autoradiography (QAR) is a useful nonspecific technique for assessing the distribution of the drug-derived radioequivalents in animals. These studies can provide information on routes of excretion, distribution or radioequivalents into target organs, as well as tissue accumulation of drug-derived radioequivalents. It must be stressed, however, that QAR is nonspecific and, thus, does not provide information on the chemical identity of the recovered radioactivity. Complementary techniques to QAR include metabolic profiling of target organs and assessment of accumulation in target tissues by exhaustive extraction [9193].

C. Humans

The need to perform in vivo human disposition studies is particularly important early in the clinical program to provide support for both in vitro and preclinical metabolism, toxicology, and pharmacology studies. Metabolic disposition studies also aid with interpretation of pharmacokinetic data, in which large intersubject variabilities are often observed [94,95]. Variabilities in hepatic levels and activities of several cytochromes P450 human enzymes have been identified [9598]. Effects contributing to these differences in human metabolism include environment, genetics (pharmacogenetics), disease state, nutritional status, gender, and age [94,99101]. Genetic variation in human cytochromes P450 has been extensively studied, with particular emphasis on the debrisoquine (CYP2D6) and mephenytoin hydroxylases (CYP2C19) [102]. Other potential polymorphisms exist, however, and the clinical significance of these effects remains to be determined [103]. Gender, race, and age [104], as well as smoking status [105] have been correlated with CYP1A2 activities in humans. Stress or clinical depression has also been found to alter CYP3A4 activities [106]. Thus, a variety of both environmental and pharmacogenetic factors can influence drug disposition and may need to be assessed early in the clinical development program because they may affect clinical response [107].

The major cytochromes P450 enzymes involved in human metabolism include CYQ1A2, CYP2D6, CYP2C8/C18, and CYP3A4 [108110]. Several noninvasive probe substrates have been proposed to assess individual cytochromes P450 activities (e.g., caffeine for CYP1A2 [111] and erythromycin for CYP3A [112,113]. Recently, questions regarding the accuracy of some in vivo probes have been raised [114]. Characterization of enzymology

and regulation involved in drug disposition should provide helpful insight and support for understanding

inter- and intrasubject variabilities. This information should also benefit the interpretation of population kinetic studies.

The assessment of enzyme induction and inhibition in humans should be examined especially as it pertains to drug interactions and toxicological consequences [115]. The effect of isoniazid, an inducer of human CYP2E1, on clorzoxazone and acetaminophen metabolism, both CYP2E1 substrates, was recently reported [116]. Drug interactions involving CYP3A4 were reported between ketoconazole and cyclosporin [117], and terfenadine and itraconzaole [118]. These examples underscore the need to understand the consequences of concurrent therapies at a molecular level [1].

Extrahepatic metabolism in humans can be an important metabolic issue. For example, the significant impact of gut metabolism via CYP3A4 on systemic availability was shown with the immunomodulator cyclosporin A [119].

At present, the metabolism scientist should consider preclinical animal data, human in vitro data, and early clinical data in designing subsequent clinical metabolism studies. For example, preclinical and clinical data showed that the b-hydroxy-b-methylglutaryl-CoA HMG-CoA reductase inhibitor simvastatin is extensively excreted in animal and human feces. These data advocated a human bile duct cannulated study to evaluate the metabolism of simvastatin [120].

IV. Metabolic Profiling

To understand the disposition of a drug candidate, a detailed profile of its metabolic fate is required. Several chromatographic techniques are at the analysts' disposal including high-performance liquid chromatography, (HPLC), gas chromatography (GC), supercritical fluid chromatography (SFC), capillary zone electrophoresis (CZE), and thin layer chromatography (TLC). Presently, reversephase HPLC is the primary chromatographic technique used for metabolite profiling. It should be noted here that microbore and nanobore HPLC chromatography are beginning to find application as alternatives to conventional HPLC.

If radiolabeled drug is lacking, metabolite profiling is typically conducted by using HPLC coupled with photodiode array ultraviolet (UV), electrochemical, or mass spectrometric detection (see below). Issues of parent drug and/or metabolite volatility and thermal stability have limited GC profiling to select studies. In the future, microbore HPLC and, to a lesser extent, CZE and SFC will see increasing use in metabolite profiling studies. The utility of tandem mass spectrometry in profiling is described later.

A. HPLC Radioactivity Detection.

An increasingly common method for metabolite profiling when radiolabeled drug is available is HPLC with on-line radioactivity detection. The use of HPLC

is dictated primarily by the fact that metabolites are typically more polar than the parent drug (and thus more water soluble). This technique was historically used with off-line fraction collection and liquid scintillation spectrometry (LSC) to follow parent and metabolite fractions. Disadvantages of this technique were many including the inability to resolve closely eluting peaks and the labor- and time-consuming nature of the process [121]. More recently, flow-through scintillation spectrometers have improved this procedure and allow for on-line monitoring of metabolite profiles [122]. These detectors monitor radioactivity by either heterogeneous or homogeneous detection systems. The first developed online systems were heterogeneous detectors. Unfortunately, sensitivity and contamination problems limited their utility. Nevertheless these systems do allow for the recovery of radiolabeled material. Homogeneous detection systems differ from heterogeneous systems in having the column effluent mixed with scintillation cocktail prior to analysis. This system is more sensitive and has fewer contamination problems.

B. Sample Preparation and Metabolite Isolation

The metabolite containing matrix should ideally be injected directly onto the chromatographic system to minimize sample degradation, formation of artifacts, and potential differences in individual component recoveries. Sometimes owing to low levels of drug and metabolites or incompatibility of the biological matrix with the chromatographic system, sample manipulation will be required. In these instances, the drug and metabolites must be either concentrated or extracted from the matrix [123,124]. Concentration techniques routinely used include evaporation or lyophilization.

Extraction methods used in sample preparation include liquid-liquid extraction (LLE) and solid phase extraction (SPE) [123,124]. LLE requires a greater solubility of the drug metabolite(s) in suitable organic solvents than in the aqueous phase. This will typically require an appropriate pH adjustment of the aqueous phase such that drug and metabolite(s) can be successfully extracted. The high degree of specificity of LLE can also be its downfall. In general, the drug is more lipophilic than its metabolites. Thus, this technique will often easily remove the drug from the matrix while leaving behind the more polar metabolites. SPE differs from LLE in that the drug and metabolite(s) are adsorbed to a bonded phase that is then washed of endogenous matrix compounds before elution of drug-derived materials with an appropriate solvent. The advantages of SPE include smaller sample volumes, cost, and speed. Major disadvantages are matrix incompatibility and column plugging.

An alternative method for sample isolation and concentration is semipreparative HPLC in which larger volumes of matrix, such as urine, can be injected directly on column.

V. Metabolite Identification

Metabolite profiling or pharmacokinetic studies may allow for the observation of drug-derived metabolites. The identification of metabolites may be accomplished using a variety of methodologies (e.g., diode array, electrochemical, or mass spectrometric detection) depending on the desired level of accuracy. For example, in early discovery tentative identification may be sufficient to address a metabolic issue, while in full-scale development, structural confirmation of metabolites is usually needed. The ability to identify metabolites, even tentatively, in the discovery phase can provide valuable information to aid in compound development (e.g., appropriate selection of toxicology species) or selection of a replacement drug). Moreover, confirmation of metabolite structure can be useful for identifying active metabolites as possible backup compounds and identifying potential toxic metabolites. The emphasis of this section will be on the newer or more widely used technologies for metabolite identification and confirmation.

A. Mass Spectrometry

Mass spectrometers provide exceptional sensitivity and selectivity and the ability to determine the mass of molecular and structurally significant fragment ions. It is not surprising, then, that mass spectrometry plays a leading role in the elucidation of drug metabolites by virtue of its ability to provide molecular weight information, elemental composition, and structure with only small quantities of sample. Indeed, as the recent trend continues toward significantly enhanced drug potency (and specificity) with the concomitant reduction in drug dose, the need for increasingly sensitive methodology will only become greater and, in all eventuality, may serve to drive the future development of mass spectrometry.

1. Mass Spectrometer Instrumentation

Mass spectrometers are available in a number of different configurations including quadrupole, magnetic sector, time-of-flight, ion trap, and ion cyclotron resonance analyzers. All mass spectrometers consist of an ion source, the mass analyzer, and the detector. The mass analyzer separates ions by virtue of their mass-to-charge ratio (m/z). Mass analyzers separate ions by making use of appropriate electric fields and, sometimes, in combination with magnetic fields.

A brief description of the more common types of instrument configurations is given below. However, it should be noted that the choice of instrument should be determined by the nature of the analyses required and that no one mass spectrometer is suitable for all types of analyses.

a. Quadrupole Mass Spectrometers The overwhelming majority of mass spectrometers in use today are quadrupole instruments. These instruments are

particularly well suited for applications requiring rapid mass scan rates at modest resolution and mass range. A quadrupole electric field is created by electrical potentials applied to four parallel rods. A mass spectrum is obtained by scanning the DC and RF voltages applied to opposite pairs of rods. The instruments can operate at moderate ion source pressures (<10-4 torr) and, thus, are frequently interfaced with ion sources that have high pressure requirements such as those, for example, that employ atmospheric pressure ionization.

b. Magnetic Sector Mass Spectrometers Most commercial magnetic sector instruments are double-focusing: by appropriate combinations of electric (E) and magnetic (B) sector fields, an ion beam that is both divergent and inhomogeneous in energy is brought into focus. Instruments in which the electric sector precedes the magnetic sector are known as forward geometry instruments; when the magnetic sector precedes the electric sector, they are known as reverse geometry instruments. The resolving power of magnetic sector instruments is significantly higher than quadrupole instruments, and can have an accuracy of mass measurement better than 1 ppm.

c. Time-of-Flight Mass Spectrometers Time-of-flight (TOF) spectrometers have seen a recent resurgence in popularity primarily because of their ability to obtain complete mass spectra over a very wide mass range (>100,000 daltons [Da]) together with the arrival of matrix-assisted laser desorption (see below), which can ionize very large molecules. The instrument is of very simple design: ions of different masses leave the ion source simultaneously, traverse a hollow flight tube, and arrive at the detector at different times according to their initial momentum.

2. Sample Ionization Methods

Gas chromatography-mass spectrometry (GC/MS) is a well-established and powerful technique widely used in profiling volatile drug metabolites. Nevertheless, most drug metabolites are either insufficiently volatile or too labile to be amenable to GC/MS analysis. HPLC is now regarded as the method of choice for the separation of drug metabolites in biological matrices. The discussions below on sample ionization methods will, therefore, be limited to those techniques amenable to liquid chromatography (LC) introduction. Several approaches to LC/MS are available commercially, including direct liquid introduction, moving belt, particle beam, thermospray, electrospray, atmospheric pressure ionization, and continuous-flow fast atom bombardment (FAB). Of these, the latter four comprise the more successful techniques and will be discussed below. Thermospray and particle beam ionization are widely used techniques that, nevertheless, have not undergone any significant development for several years. In the interest of discussing here only those recent developments in sample ionization methods, the

interested reader is directed to articles on these two techniques [125131].

a. Continuous-Flow Fast Atom Bombardment/Liquid Secondary Ion Mass Spectrometry Desorption ionization using FAB or liquid secondary ion mass spectrometry (LSIMS) is a standard method for the analysis of thermally labile and nonvolatile compounds [132]. FAB has been combined on-line with reversed-phase HPLC in a system called continuous-flow or dynamic FAB [133]. In this system, reversed-phase HPLC mobile phase, containing 1% glycerol as matrix, is introduced into the ion source via a steel frit. As an alternative, the FAB matrix can be added at the FAB probe tip by using coaxial-flow addition [134]. The sample and matrix are then ionized on the inner surface of the frit with a beam of accelerated argon or xenon atoms or cesium ions. A significant benefit of the technique is that the chemical background associated with static FAB is considerably less for dynamic FAB. The chemical background consists of noncorrelated ions of unknown structure and chemical composition thought to arise from degradation of the matrix and sample by the bombardment of the atom or ion beam. In contrast, for dynamic FAB the eluting matrix is present at the tip for only a short period and is soon replaced by fresh solvent, leading to a steady state and improved long-term stability with reduced chemical background.

The optimum rate at which the HPLC mobile phase can be introduced into the ion source is about 5 μl/min and this clearly requires the use of a reliable splitter when conventional 25 mm bore HPLC columns are used [135]. Although a commercial post-column splitter is available, in practice it is much simpler and more reliable when capillary HPLC columns are used, since good resolution and an acceptable speed of analysis can be achieved with flow rates of 5 μl/min [136]. To minimize band broadening caused by extracolumn dead volume, coaxial-flow addition of FAB matrix to the mobile phase is normally carried out at the FAB probe tip.

The application of FAB to the identification of drug metabolites was reviewed [137]. Examples reviewed include the identification of glucuronide conjugates, sulfate esters, mercapturic acid conjugates, amino acid conjugates, and bile salt conjugates.

b. Electrospray Ionization Electrospray ionization mass spectrometry (ESI-MS) has become an important analytical technique for chemical, biochemical, and biological materials [138]. The area of most success and application is in the identification of large biomolecules [139]. The transfer of ions from solution to the gas phase is a desolvation process by which little if any extra internal energy is imparted to the ions. Although the mechanism of electrospray ionization is not completely understood, there are four major processes in ESI-MS: the production of charged droplets from electrolyte dissolved in a solvent; shrinkage of charged droplets by solvent evaporation and repeated droplet disintegrations (fissions), leading ultimately to very small, highly charged droplets capable

of producing gas-phase ions; the mechanism of gas-phase ion produc-

tion; and secondary processes, by which gas-phase ions are modified in the atmospheric and the ion sampling regions of the spectrometer [140]. From a practical standpoint, low electrolyte concentrations, routinely in the 10^{-5}-10^{-3} M range, are required for the operation of ESI [141,142]. Using water as the solvent can likewise lead to the initiation of an electric discharge from the capillary tip, particularly in the negative ion mode unless traces of discharge suppressing gases such as SF_6 or larger quantities of O_2 are added to the air surrounding the ES capillary [143,144].

Collision-induced dissociation (CID) of analyte ions can be obtained through acceleration of sample ions in the first vacuum region (nozzle-skimmer region). At low collision energy, (e.g., a voltage drop of 45 V), residual solvent molecules clustered on the sample ions can be removed. At higher collision energies (150250V), sample ions may be induced to fragment to give rise to structurally significant fragment ions. The method, however, does not offer the precursor ion specificity of CID experiments performed in a tandem mass spectrometer (see below). The resultant fragment ion spectra often contain interfering fragment ions generated from chemical background in the ion source of solvents, or from coeluting endogenous components.

ESI has emerged as the method of choice for the analysis of large peptides and glycopeptides [138,145147]. Although CF-FAB is more often chosen for lower-molecular-weight peptides, FAB is not particularly amenable to the analysis of peptides of molecular weights of over 3000 Da [149]. ESI has shown application in other areas of pharmaceutical relevance including the analysis of toxins, b-lactam antibiotics, aminoglycosides, nucleoside bases, sulfamides, steroids, and glycerides [150153]. A recent application of ESI was the detection of protein-protein and protein-ligand noncovalent interactions [154156]. This application of ESI has significant implications for the study of drug-substrate interactions. The ability to detect and measure noncovalent complexes may eventually provide a means to probe the active site for binding of xenobiotics and xenobiotic structure-function relationships. Moreover, the strength of that binding may be investigated by subsequent collisional activated decomposition MS/MS.

c. Atmospheric Pressure Ionization Atmospheric pressure ionization (API) is performed by using either a heated nebulizer or a pneumatically assisted electrospray (ion-spray) interface. Both interfaces involve soft ionization processes that generate primarily molecular ions. The heated nebulizer interface is compatible with conventional LC flow rates. A heated (100°C) makeup gas carries the LC effluent through a heated tube that vaporizes the droplets and the analytes in the gas stream before flowing into the reaction region of the source. In the reaction region, the sample is chemically ionized by charge transfer from reagent ions (e.g., H_3O^+ or O^-) formed by gentle corona discharge of the

solvent vapor. The ion-spray interface consists of a coaxial pneumatic nebulizer held

at high electrical potential, typically 46 kV [157]. The chromatographic effluent is introduced at low flow rates (20200 μl/min) through a capillary to the end of the nebulizer where it is exposed to the high electrical potential and nebulizer gas. By a process of ion evaporation, preformed ions present in solution are released from rapidly shrinking, highly charged droplets.

API is also a suitable interface for coupling other separation techniques including supercritical fluid chromatography, capillary electrophoresis, and ion chromatography [158160]. Moreover, for many pharmaceutical applications of the LC/API interface, the API interface has displayed performance clearly superior to that of the older thermospray interface [161,162]. A recent review [162] of pharmaceutical, biomedical, and drug metabolism applications of API demonstrates the increasing acceptance of the technique. This is in no small part because of the recent commercial introduction of dedicated API (and electrospray) instruments.

API has shown comparable potential to ESI for the detection and measurement of noncovalent complexes. The application of API for the detection of noncovalent complexes of biological macromolecules was reviewed recently [163].

3. Recent Advances in Sample Ionization/Sample Introduction

a. Capillary Electrophoresis/Mass Spectrometry Capillary electrophoresis (CE) is a liquid-phase separation technique capable of highly efficient separations of samples of biological origin. Separations with plate numbers more than 10^6 are possible [164]. Typical flow rates from the CE separation capillary are between 0 and 100 nl/min. These low flow rates present a challenge for successfully coupling CE to the mass spectrometer. Three CE/MS interfaces are, however, currently available: continuous-flow FAB, electrospray, and ion spray. The simple solution to the problem of coupling the low flow rates of CE for each of these interfaces was to use postcapillary addition of a make-up liquid [165167]. The high resolving power of CE also places constraints on the type of mass spectrometer instrumentation capable of acquiring a full mass spectrum where peak widths require scan times of at least 1 s over a mass range of 20020000 Da. Nevertheless, CE/MS has proven to be an excellent qualitative and quantitative tool for the analysis of primarily peptides and proteins [168], although the method was applied to the analysis of macrolide antibiotics [169].

b. Matrix-Assisted Laser Desorption/Ionization The technique of matrixassisted laser desorption/ionization (MALDI) combined with time-of-flight mass spectrometry has significantly extended the accessible mass range for the analysis of bioorganic molecules into the range of several hundred thousand daltons [170]. In a remarkable discovery, it

was observed that small, non-UV-absorbing molecules could be successfully desorbed intact if they were immersed in a suitably absorbing matrix [171,172]. Subsequent investigation demonstrated the

utility of the technique to desorb large molecules. Moreover, desorption of even small peptides and glycopeptides is possible despite the presence of significant ubiquitous biological residue. Among the many organic matrices suitable for MALDI, nicotinic and sinapinic acids appear to give the best results at UV wavelengths of 266 nm. Proper sample preparation is essential for good results. Sample homogeneity is critical and the addition of additives such as trifluoracetic acid or ethanol can be beneficial [173].

Under certain conditions, MALDI can produce intact molecular ions of proteins consisting of noncovalently bound subunits [174].

4. Tandem Mass Spectrometry

The soft ionization methods so often employed in the analysis of biomolecules give rise to only molecular ions and very few structurally significant fragment ions. Moreover, whatever the type of separation technique employed in isolating drug metabolites, ubiquitous chemical contamination will be present that obscures the presence of the metabolite in a mass spectrum. A solution to each of these issues is the application of tandem mass spectrometry. The mass spectrometer mass separates an analyte ion from the chemical contamination, and by employing collisional activation a mass spectrum of structurally significant fragment ions may be obtained from the mass separated ion. The product ion spectrum so produced is but one type of experiment available to tandem mass spectrometry. Constant neutral loss scans and precursor ions scans are also possible and should not be overlooked, particularly when the identity of a drug metabolite is unknown.

a. Instrumentation The choice of instrumentation suitable for MS/MS experiments is limited only by the size of your budget. At one end of the instrument spectrum are triple quadrupole mass spectrometers that use the second quadrupole in the series as an efficient device for the containment, activation, and transmission of mass selected ions. This is accomplished by operating the quadrupole with only RF potentials applied to pairs of opposite rods. The first and third quadrupoles are used as mass analyzers to mass select a precursor ion and mass analyze product ions, respectively [175].

Tandem magnetic sector instruments incorporate two, three, or four sectors for mass selection and analysis. Four-sector configurations with array detection represent the state of the art in tandem mass spectrometry. They allow mass selection of precursor ions at very high resolution and mass analysis of product ions at resolving powers of at least 5001000 [176].

The combination of quadrupoles with magnetic sector mass spectrometers gives rise to hybrid instruments. Hybrid instruments feature a high-performance stage (magnetic

sector), the availability of high- and low-energy CAD MS/MS experiments, and the possibility of MS/MS/MS experiments. The various configurations and scan modes of hybrid instruments have been reviewed [177].

Of particular importance are the two scan modes constant neutral loss (CNL) and multiple reaction monitoring (MRM). On a hybrid instrument, CNL scans are obtained by simultaneously scanning the magnet and quadrupole mass filter with a constant mass offset; the electrostatic analyzer(s) are static. The fragmentation of precursor ions occurs in the rf quadrupole. MRM experiments are essentially product ion MS/MS spectra but with only selected precursor-product ions being monitored.

b. Metabolite Mapping The technique known as metabolite mapping is a powerful method for rapidly screening for and identifying metabolites of a xenobiotic compound in complex in vivo and in vitro biological matrices [178180]. The strategy normally involves constant neutral loss scans that monitor a compound class-selective fragmentation [180,181].

A general procedure for using tandem mass spectrometry in metabolite analysis begins with a preliminary study of the parent drug to determine key substructural characteristics that may be used as unique markers. The structural characteristics of the parent drug are best probed by obtaining product ion MS/MS spectra using a combination (if available) of high- and low-energy collisional activation. Following identification of several structurally significant product ions a series of precursor ion or, more usually, constant neutral loss scans are then performed to screen for unknown metabolites.

c. Isotope Dilution Isotope dilution (cluster) is a technique routinely used in mass spectrometry to aid in the identification of drug metabolites [182,183]. Although stable isotopes are more often used in routine mass spectrometric analyses, their incorporation in MS/MS experiments provides enhanced specificity and selectivity for the identification and quantification of drug metabolites. The isotope-cluster technique typically uses a mixture of labeled and unlabeled compound to obtain an artificial isotope pattern in its mass spectrum that contains a doublet with a mass difference determined by the abundance of label. The artificial isotope pattern is exploited in MS/MS by using either constant neutral loss scans or multiple reaction monitoring that now profiles two transitions: fragmentation of labeled and unlabeled metabolite. For ethical reasons, on occasion, it may be more appropriate to use a stable label rather than a radioactive labeled drug for in vivo studies and to use an MS detection protocol.

B. Nuclear Magnetic Resonance

The arrival of pulse methods with Fourier transformation, two-dimensional techniques, and higher field superconducting magnets, along with advances in computing technology, have expanded the application of nuclear magnetic resonance (NMR) to drug metabolism. NMR has found application in the characterization of isolated metabolite fractions,

analysis of metabolites in unprocessed matrices, and tissue extracts. In addition, noninvasive applications of NMR include ob-

taining spectra (magnetic resonance spectroscopy [MRS]) and images, (magnetic resonance imaging [MRI]) in vivo. In the area of drug metabolism, the most useful NMR-observable nuclei include ^{1}H, ^{13}C, and ^{19}F. The most sensitive of these nuclei is ^{1}H followed by ^{19}F (80% of ^{1}H), both of which are found in approximately 100% natural abundance. Carbon 13 studies are most useful when the natural abundance of approximately 1% is increased by chemical enrichment. Fluorine-19-containing drug candidates are especially useful in drug metabolism studies due to high sensitivity and the lack of endogenous background [184186]. Readers with limited NMR experience who wish to increase their understanding of modern NMR techniques are referred to other reviews on the subject [187,188]. The utility of NMR in drug metabolism studies has been described [184,186,189,190]. In the area of metabolite characterization, the regiochemical information afforded by NMR makes it a powerful and valuable tool. NMR has seen application in studies of in vitro and ex vivo (e.g., perfusates from organ perfusion) metabolism, and in matrices obtained from in vivo studies (plasma, urine, bile, and tissue extracts).

Proton NMR can be used for the nonselective identification of drug and metabolites as well as endogenous components of a matrix [184,186,189,191193]. Thus, NMR can not only give valuable information on drug-derived material but also provide information on the physiological make-up of the matrix. Solvent suppression problems associated with water in the matrix can be addressed, in some cases, by lyophilization or by specific water-attenuating pulse sequences [194]. Solid phase extraction was used successfully as a minimalistic chromatographic separation, before NMR analysis, for the urinary analysis of drugs including ibuprofen, paracetamol, aspirin, oxpentifylline, naproxen, and an antiatherosclerotic drug candidate CI-976 [195,196].

Directly linked HPLC to a dedicated high field NMR instrument was used to monitor and identify both endogenous and drug-derived metabolites in human urine following oral administration of ibuprofen [197,198]. The utility of MRS [185,186] and MRI [185,148] as noninvasive techniques for following drug and metabolites in vivo has been reviewed using ^{19}F-containing drugs in animals and humans. It must be mentioned, however, that these compounds are administered in very high doses, while the bulk of drugs under development are dosed at levels below the detection limits of these techniques. Although there may be more applications of MRS and MRI in drug metabolism as the technology improves, at this time these experiments are better suited to investigating the effect of the drug on the biological system.

VI. Future Directions

The field of drug metabolism has changed significantly over the last 5 years and will

continue to evolve dramatically as we approach the year 2000 and beyond.

Ultimately, the successful discovery and development of new drugs will require careful and well-planned metabolism studies. The need to conserve resources while retaining the competitive edge will drive the development and use of in vitro and bioanalytical methodologies. The use of expressed enzymes, not only cytochromes P450, will be expanded and more appropriately applied. Structure-function studies will allow better understanding of metabolizing enzyme substrate binding and catalysis properties. Monoclonal antibodies for drug metabolism research should become more readily available along with more potent and selective inhibitors and modulators of drug-metabolizing enzymes. Issues of radioactivity use will influence drug metabolism and place more emphasis on stable label studies and other, alternative methods for drug-derived recovery studies. The expanding field of pharmacogenetics and the realization of inter-subject variabilities will also have an impact on clinical metabolism studies. Phenotyping of subjects will expand as more selective probe substrates become available.

We hope that the emphasis placed in this chapter on new and emerging technologies in drug metabolism, while not all inclusive, has provided insight into the role that state of the art drug metabolism can play in the overall drug discovery and development process.

References.

1. C. C. Peck, R. Temple, and J. M. Collins. Understanding consequences of concurrent therapies. JAMA 269:15501152, 1993.

2. D. A. Smith and B. C. Jones. Speculations on the substrate structure-activity relationship (SSAR) of cytochrome P450 enzymes. Biochem. Pharmacol. 44:20892098, 1992.

3. K. R. Korzekwa and J. P. Jones. Predicting the cytochrome P450 mediated metabolism of xenobiotics. Pharmacogenetics 3:118, 1993.

4. S. L. Kelly. P-450 biotechnology. Biochem. Soc. Trans. 21:10151017, 1993.

5. M. H. Tarbit, M. K. Bayliss, D. Herriott, S. R. Hood, J. L. Hutson, G. R. Park, and C. J. Serabjit-Singh. Applications of molecular biology and in vitro technology to drug metabolism studies: An industrial perspective. Biochem. Soc. Trans. 21:10181023, 1993.

6. M. J. Fasco, J. B. Silkworth, D. A. Dunbar, and L. S. Kaminsky. Rat small intestinal cytochromes P450 probed by warfarin metabolism. Mol. Pharmacol. 43:226233, 1993.

7. A. Ward, D. Hickman, J. W. Gordon, and E. Sim. Arylamine N-transferase in human red blood cells. Biochem. Pharmacol. 44:10991104, 1992.

8. D. R. Nelson, T. Kamataki, D. J. Waxman, F. P. Guengerich, R. W. Estabrook, R.

Feyereisen, F. J. Gonzalez, M. J. Coon, I. C. Gunsalus, O. Gotoh, K. Okuda, and D. W. Nebert. The P450 superfamily: Update on new sequences, gene mapping, accession numbers, early trivial names of enzymes and nomenclature. DNA Cell Biol. 12:151, 1993.

9. F. Gonzalez. The molecular biology of cytochromes P450s. Pharmacol. Rev. 40:243288, 1989.

10. E. Mosialou, C. Andersson, G. Lundqvist, G. Andersson, T. Bergman, H. Jornvall, and R. Morgenstern. Human liver microsomal glutathione transferase. FEBS 315:7780, 1993.

11. F. P. Guengerich, G. A. Dannan, S. T. Wright, and M. V. Martin. Purification and characterization of microsomal cytochrome P450s. Xenobiotica 12:701716, 1982.

12. P. J. Sabourin, B. P. Smyfer, and E. Hodgson. Purification of flavin-containing monooxygenase from mouse and pig liver microsomes. Int. J. Biochem. 16:713720, 1984.

13. W. B. Jakoby (Ed.). Methods in Enzymology, Vol. 77, Detoxication and Drug Metabolism: Conjugation and Related Systems. Academic, Press, New York, 1981.

14. K. Oeda, T. Sakaki, and H. Ohkawa. Expression of rat liver cytochrome P450 MC cDNA in *Saccharomyces cerevisiae.* DNA 4:203210, 1985.

15. H. J. Ahr, P. Beaune, J. Dohmer, and F. J. Weibel (Eds.). Applications of Cellular Systems in Drug Metabolism and Toxicity StudiesToxicology 82. Elsevier, Ireland, 1993.

16. J. Doehmer and H. Greim. Cytochromes P450 in genetically engineered cell cultures: the gene technological approach. In Handbook of Experimental Pharmacology, Cytochrome P450, Vol. 105. Edited by G. V. R. Born, P. Cuatrecases, and H. Herken. Springer-Verlag, Berlin, 1993, pp. 415429.

17. N. Lomri, Z. Yang, and J. R. Cashman. Expression in *Escherichia coli* of the flavin-containing monooxygenase D (form II) from adult human liver: Determination of a distinct tertiary amine substrate specificity. Chem. Res. Toxicol. 6:425429, 1993.

18. B. Burchell and M. W. H. Coughtrie. UDP-glucuronosyltransferases. Pharmacol. Ther. 43:261289, 1989.

19. D. M. Otterness, E. D. Wieben, T. C. Wood, R. W. G. Watson, B. J. Madden. D. J. McCornmick, and R. M. Weinshilboum. Human liver dehydroepiandrosterone sulfotransferase: Molecular cloning and expression of cDNA. Mol. Pharmacol. 41:865872, 1992.

20. H.-P. Eugster, C. Sengstag, A. Hinnen, U. Meyer, and F. E. Wurgler. Heterologous expression of human microsomal epoxide hydrolase in *Saccharomyces cerevisiae,* study of the valpromide-carbamazepine epoxide interaction. Biochem. Pharmacol. 42:13671372, 1991.

21. A. J. Townsend, C.-P. D. Tu, and K. H. Cowan. Expression of human mu and alpha class glutathione S-transferases in stably transfected human MCF-7 breast cancer cells: Effect on cellular sensitivity to cytotoxic agents. Mol. Pharmacol. 41:230236, 1992.

22. S. Ohsako and T. Deguchi. Cloning and expression of cDNAs for polymorphic and monomorphic arylamine N-acetyltransferases from human liver. J. Biol. Chem. 265:46304634, 1990.

23. R. P. Remmel and B. Burchell. Validation and use of cloned, expressed human drug-megabolizing enzymes in heterologous cells for analysis of drug metabolism and drug-drug interactions. Biochem. Pharmacol. 46:559566, 1993.

24. F. P. Guengerich. Analysis and characterization of enzymes. Principles and Methods of Toxicology, 2nd ed (A. W. Hayes, ed.), Raven Press, New York, 1989, pp. 777814.

25. B. A. Fowler, G. W. Lucier, and A. W. Hayes. Organelles as tools. Principles and methods of toxicology, 2nd ed (A. W. Hayes, ed.), Raven Press, New York, 1989, pp. 815833.

26. L. K. Low and N. Castagnoli, Jr. Metabolic changes of drugs and related organic compounds. Organic Medicinal and Pharmaceutical Chemistry, 8th ed. (R. F. Doerge ed.), Lippincott, Philadelphia, 1982, pp. 55127.

27. F. Berthou, B. Guillois, C. Riche, Y. Dreano, E. Jacqz-Augraub, and P. H. Beaune. Interspecies variations in caffeine metabolism related to cytochrome P4501A enzymes. Xenobiotica 22:671680, 1992.

28. C. Mani, H. V. Gelboin, S. S. Park, R. Pearce, A. Parkinson, and D. Kupfer. Metabolism of the antimammary cancer antiestrogenic agent tamoxifen: Cytochrome P-450 catalyzed N-demethylation and 4- hydroxylation. Drug Metab. Dispos. 21:645656, 1993.

29. C. Mani, E. Hodgson, and D. Kupfer. Metabolism of the antimammary cancer antiestrogenic agent tamoxifen. II. Flavin-containing monooxygenase-mediated *N*-oxidation. Drug Metab. Dispos. 21:657661, 1993.

30. R. W. Wang, P. H. Kari, A. Y. H. Lu, P. E. Thomas, F. P. Guengerich, and K. P. Vyas. Biotransformation of lovastatin IV. Identification of cytochrome P450 3A proteins as the major enzymes responsible for the oxidative metabolism of lovastatin in rat and human liver microsomes. Arch. Biochem. Biophys. 290:355361, 1991.

31. S. Madden, T. F. Woolf, W. F. Pool, and B. K. Park. An investigation into the formation of stable, protein-reactive and cytotoxic metabolites from tacrine in vitro: studies with human and rat liver microsomes. Biochem. Pharmacol. 46:1320, 1993.

32. M. D. Tingle and B. K. Park. The use of a three compartment in vitro model to investigate the role of hepatic drug metabolism in drug-induced blood dyscrasias. Br. J. Clin. Pharmacol. 36:3138, 1993.

33. K. E. Thummel, E. D. Kharasch, T. Podoll, and K. Kunze. Human liver microsomal enflurane defluorination catalyzed by cytochrome P-450 2E1. Drug Metab. Dispos. 21:350357, 1993.

34. S. Ponchaut, F. van Hoof, and K. Veitch. In vitro effects of valproate and valproate metabolites on mitochrondrial oxidations relevance of CoA sequestration to the observed inhibitions. Biochem. Pharmacol. 43:24352442, 1992.

35. R. C. Tukey and K. J. Cameron. Side-chain specificities of human and bovine

cytochromes P-450scc. Eur. J. Biochem. 217:209215, 1993.

36. L. R. Schwarz and F. J. Wiebel. Cytochrome P450 in primary and permanent cell cultures. Handbook of Experimental Pharmacology, Cytochrome P450, Vol. 105 (G. V. R. Born, P. Cuatrecasas, and H. Herken, eds.), Springer-Verlag, Berlin, 1993, pp. 415429.

37. M. N. Berry, A. M. Edwards, and G. J. Barritt. Isolated hepatocytes preparation, properties and applications. Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 21 (R. H. Burdon, and P. H. van Knippenberg, eds), Elsevier, Amsterdam, 1991, pp. 158.

38. P. O. Seglen. Isolation of hepatocytes by collagenase perfusion. Methods in Toxicology, Vol. 1, Part A, In Vitro Biological Systems (C. A. Tyson and J. M. Frazier, eds.), Academic Press, New York, 1993, pp. 231278.

39. V. Rogiers and A. Vercruysse. Rat hepatocyte cultures and co-cultures in biotransformation studies of xenobiotics. Toxicology 82:193208, 1993.

40. L. Pichard, I. Fabre, G. Fabre, J. Domergue, B. Saint Aubert, G. Mourad, and P. Maurel. Predicting drug interactions using cultured human hepatocytes. Hepatology 14:396398, 1991.

41. A. Guillouzo, P. Gripon, D. Ratanasavanh, B. Clement, and C. Guguen-Guillouzo. Cultured human hepatocytes as a model system for man in pharmacotoxicological research. Advances in Applied Toxicology (Dayan, A. D. and A. J. Paine, eds.), Taylor and Francis, London, 1989, pp. 165190.

42. J. Zaleski, J. Richburg, and F. C. Kauffman. Preservation of the rate and profile of xenobiotic metabolism in rat hepatocytes stored in liquid nitrogen. Biochem. Pharmacol. 46:111116, 1993.

43. C. Chesné, C. Guyomard, A. Fautrel, M.-G. Poullain, B. Frémond, H. De Jong, and A. Guillouzo. Viability and function in primary culture of adult hepatocytes from various animal species and human beings after cryopreservation. Hepatology 18:406414, 1993.

44. J. A. Coundouris, M. H. Grant, J. Engeset, J. C. Petrie, and G. M. Hawksworth. Cryopreservation of human adult hepatocytes for use in drug metabolism and toxicity studies. Xenobiotica 23:13991409, 1993.

45. E. G. Schuetz, J. D. Schuetz, S. C. Strom, M. T. Thompson, R. A. Fisher, D. T. Molowa, D. Li, and P. S. Guzelian. Regulation of human liver cytochromes P-450 in family 3A in primary and continuous culture of human hepatocytes. Hepatology 18:12541262, 1993.

46. A. Guillouzo, F. Morel, O. Fardel, and B. Meunier. Use of human hepatocyte cultures for drug metabolism studies. Toxicology 82:209219, 1993.

47. C. L. Krumdieck, J. E. dos Santos, and K.-J. Ho. A new instrument for the rapid preparation of tissue slices. Anal. Biochem. 104:118123, 1980.

48. K. Brendel, A. J. Gandolfi, C. L. Krumdieck, and P. F. Smith. Tissue slicing and culturing revisited. Trends Pharmacol. Sci. 8:1115, 1987.

49. P. F. Smith, G. Krack, R. L. McKee, D. G. Johnson, A. J. Gandolfi, V. J. Hruby, C. L. Krumdieck, and K. Brendel. Maintenance of adult rat liver slices in dynamic organ culture. In Vitro Cell. Dev. Biol. 22:706712, 1986.

50. J. Barr, A. J. Weir, K. Brendel, and I. G. Sipes. Liver slices in dynamic organ culture. II. An in vitro cellular technique for the study of integrated drug metabolism using human tissue. Xenobiotica 21:341350, 1991.

51. K. Brendel, R. L. Fisher, C. L. Krumdieck, and A. J. Gandolfi. Precision-cut rat liver slices in dynamic organ culture for structure-toxicity studies. Methods in Toxicology, Vol. 1, Part A. In Vitro Biological Systems (C. A. Tyson and J. M. Frazier, eds.), Academic Press, New York, 1993, pp. 222230.

52. A. E. M. Vickers, S. Connors, M. Zollinger, W. A. Biggi, A. Larrauri, J. P. W. Vogelaar, and K. Brendel. The biotransformation of the ergot derivative CQA 206291 in human, dog, and rat liver slice cultures and prediction of in vivo plasma clearance. Drug Metab. Dispos. 21:454459, 1993.

53. F. Berthou, D. Ratanasavanh, C. Riche, D. Picart, T. Voirin, and A. Guillouzo. Comparison of caffeine metabolism by slices, microsomes and hepatocyte cultures from adult human liver. Xenobiotica 19:401417, 1989.

54. P. F. Smith, R. Fisher, P. J. Shubat, A. J. Gandolfi, C. L. Krumdieck, and K. Brendel. In vitro cytotoxicity of allyl alcohol and bromobenzene in a novel organ culture system. Toxicol. Appl. Pharmacol. 87:509522, 1987.

55. M. G. Miller, J. Beyer, G. L. Hall, L. A. DeGraffenried, and P. E. Adams, Predictive value of liver slices for metabolism and toxicity in vivo: Use of acetaminophen as a model hepatotoxicant. Toxicol. Appl. Pharmacol. 122:108116, 1993.

56. T. Prueksaritanont, M. A. Correia, A. E. Rettie, D. C. Swinney, P. E. Thomas, and L. Z. Benet. Cyclosporine metabolism by rat liver microsomes: Evidence for involvement of enzyme(s) other than cytochromes P-450 3A. Drug Metab. Dispos. 21:730737, 1993.

57. L. Ganawardhana, J. Barr, A. J. Weir, K. Brendel, and I. G. Sipes. The N-acetylation of sulfamethazine and p-aminobenzoic acid by human liver slices in dynamic organ culture. Drug Metab. Dispos. 19:648654, 1991.

58. G. M. Powell, H. M. Hughes, and C. G. Curtis. Isolated perfused liver technology in metabolic and toxicological problems. Drug Metab. Drug Interact. 7:5486, 1989.

59. R. S. Jones. Liver perfusion techniques in toxicology. Biochemical Toxicology: A Practical Approach (K. Snell and B. Mullock, eds.), IRL Press, Oxford, 1987, pp. 2356.

60. S. M. Khedun, B. Jaharaj, W. P. Leary, and T. Naicker. The effect of therapeutic doses of paracetamol on liver function in the rat perfused liver. J. Pharm. Pharmacol. 45:566569, 1993.

61. G. Gonzalez-Martin, G. Ponce, V. Inostroza, M. Gonzalez, C. Paulos, and A. Guevara. The disposition of nifurtimox in the rat isolated perfused liver: Effect of dose size. J. Pharm. Pharmacol. 45:7274, 1993.

62. R. J. Graves and P. F. Swann. Clearance of N-nitrosodimethylamine and N-nitrosodiethylamine by the perfused rat liver. Biochem. Pharmacol. 45:983989, 1993.

63. G. W. Mihaly, H. Scott, M. K. Hyman, D. J. Morgan, and R. A. Smallwood. Quinine impairs quinidine clearance in rat perfused liver. J. Pharm. Pharmacol. 45:215217, 1993.

64. D. Brewster, R. S. Jones, and D. V. Parke. The metabolism of cyclohexanecarboxylic acid in the isolated perfused rat liver. Xenobiotica 7:601609, 1977.

65. A. B. Okey. Enzyme induction in the cytochrome P450 system. Pharmacol. Ther. 45:241298, 1990.

66. B. Testa and P. Jenner. Inhibitors of cytochrome P-450s and their mechanism of action. Drug Metab. Rev. 12:1117, 1981.

67. K. W. Bock, W. Lilienblum, G. Fischer, G. Schirmer, and B. S. Bock-Hennig. Induction and inhibition of conjugating enzymes with emphasis on UDP-glucuronyltransferases. Pharmacol. Ther. 33:2327, 1987.

68. J. C. Kraner, J. M. Lasker, G. B. Corcoran, S. D. Ray, and J. L. Raucy. Induction of P4502E1 by acetone in isolated rabbit hepatocytes, role of increased protein and mRNA synthesis. Biochem. Pharmacol. 45:14831492, 1993.

69. D. Ratanasavahn, F. Berthou, Y. Dreano, P. Mondine, A. Buillouzo, and C. Riche. Methylcholanthrene but not phenobarbital enhances caeffeine and theophylline metabolism in cultured adult human hepatocytes. Biochem. Pharmacol. 39:8594, 1990.

70. L. Pichard, J. M. Fabre, J. Domergue, G. Fabre, B. Saint-Aubert, G. Mourad, and P. Maurel. Molecular mechanism of cyclosporine A drug interactions: Inducers and inhibitors of cytochrome P450 screening in primary cultures of human hepatocytes. Transplant. Proc. 23:978979, 1991.

71. A. Madan, A. Parkinson, and M. D. Faiman. Role of flavin-dependent monooxygenases and cytochrome P450 enzymes in the sulfoxidation of S-methyl N,N-dithylthiocarbamate. Biochem. Pharmacol. 46:22912297, 1993.

72. T. Prueksaritanont, M. A. Correia, A. E. Rettie, D. C. Swinney, P. E. Thomas, and L. Z. Benet. Cyclosporine metabolism by rat liver microsomes, evidence for involvement of enzyme(s) other than cytochromes P-450 3A. Drug Metab. Dispos. 21:730737, 1993.

73. F. P. Guengerich. Mechanism-based inactivation of human liver microsomal cytochrome P-450 IIIA4 by gestodene. Chem. Res. Toxicol. 3:363371, 1990.

74. D. Sesardic, A. R. Boobis, B. P. Murray, S. Murray, J. Segura, R. De La Torre, and D. S. Davies. Furafylline is a potent and selective inhibitor of cytochrome P450IA2 in man. Br. J. Clin. Pharmacol. 29:651663, 1990.

75. J. H. T. M. Ploemen, B. Van Ommen, J. J. P. Bogaards, and P. J. Van Bladeren. Ethacrynic acid and its glutathione conjugate as inhibitors of glutathione S-transferases. Xenobiotica 23:913923, 1993.

76. G. M. Powell. Alternatives to whole animals in xenobiotica research. Metabolism of Xenobiotics (J. W. Gorrod, H. Oelschlager, and J. Caldwell, eds.), Taylor & Francis, London, 1988, pp. 277284.

77. L. E. Martin and E. Reid. Isolation of drug metabolites. Progress in Drug Metabolism, Vol. 6 (J. W. Bridges and L. F. Chasseaud, eds.), Wiley, New York, 1981, pp. 197248.

78. L. G. Dring. Methods for studying metabolism and distribution in vivo of radiolabelled drugs. Biochemical Toxicology: A Practical Approach (K. Snell and B. Mullock, eds.), IRL Press, Oxford, 1987, pp. 321.

79. T. R. Browne, G. K. Szabo, A. Ajami, and D. Wagner. Performance of human mass balance/metabolite identification studies using stable isotope (13C,15N) labeling and continuous-flow isotope-ratio mass spectrometry as an alternative to radioactive labeling methods. J. Clin. Pharmacol. 33:246252, 1993.

80. G. Mikus, C. Fischer, B. Heuer, C. Langen, and M. Eichelbaum. Applications of stable isotope methodology to study the pharmacokinetics, bioavailability and metabolism of nitrendipine after i.v. and p.o. administration. Br. J. Clin. Pharmacol. 24:561569, 1987.

81. Z.-X. Xu and S. Melethil. Simultaneous sampling of blood, bile, and urine in rats for pharmacokinetic studies. J. Pharmacol. Methods 24:203208, 1990.

82. D. J. Waxman and L. Azaroff. Phenobarbital induction of cytochrome P-450 gene expression. Biochem. J. 281:577592, 1992.

83. S. Tuntaterdtum, I. P. Chaudhary, M. Cibull, L. W. Robertson, and R. A. Blouin. Acetaminophen hepatotoxicity: Influence of phenobarbital and b-naphthoflavone

treatment in obese and lean Zucker rats. Toxicol. Appl. Pharmacol. 123:219225, 1993.

84. R. G. Bars, D. R. Bell, and C. R. Elcombe. Induction of cytochrome P450 and peroxisomal enzymes by clofibric acid in vivo and in vitro. Biochem. Pharmacol. 45:20452053, 1993.

85. M. Tsutsumi, J. M. Lasker, T. Takahashi, and C. S. Lieber. In vivo induction of hepatic P4502E1 by ethanol: Role of increased enzyme synthesis. Arch. Biochem. Biophys. 304:209218, 1993.

86. V. Kobliakov, N. Popova, and L. Rossi. Regulation of expression of the sex-specific isoforms of cytochrome P-450 in rat liver. Eur. J. Biochem. 195:585591, 1991.

87. P. T. Liu, C. Ioannides, J. Shavila, A. M. Symons, and D. V. Parke. Effects of ether anaesthesia and fasting on various cytochromes P450 of rat liver and kidney. Biochem. Pharmacol. 45:871877, 1993.

88. A. Toda, S. Ogata, A. Nagamatsu, and H. Shimeno. Cytochrome P-450 in liver microsomes of streptozotocin-induced diabetic rats: Purification and characterization. Biochim. Biophys. Acta 1141:245252, 1993.

89. C. Sato and N. Izumi. Mechanism of increased hepatotoxicity of acetaminophen by the simultaneous administration of caffeine in the rat. J. Pharmacol. Exp. Ther. 248:12431247, 1989.

90. A. R. Boobis, D. Sesardic, B. P. Murray, R. K. Edwards. A. M. Singleton, K. J. Rich, S. Murray, R. De La Torre, J. Segura, O. Pelkonen, M. Pasanen, S. Kobayashi, T. Zhi-Guang, and D. S. Davis. Species variation in the response of the cytochrome P-450-dependent monooxygenase system to inducers and inhibitors. Xenobiotica 20:11391161, 1990.

91. W. McNally, W. Pool, P. DeHart, B. Hanson, T. Woolf, and T. Chang. Distribution of 14C-tacrine radioequivalents in 3-methylcholanthrene induced rats after a single oral dose: autoradiographic and metabolite profiling studies. ISSX Proc. 2:165, 1992.

92. W. P. McNally, W. F. Pool, P. DeHart, B. Hanson, and T. F. Woolf. Effect of multiple dose administration on distribution of [14C]tacrine in rats: quantitative autoradiographic (QAR) and metabolic analysis. Proc. ISSX 4:210, 1993.

93. W. B. McNally, P. DeHart, W. Pool, and T. Chang. A comparison of in vivo distribution of radioactivity in rat brain following oral administration of tacrine or its 1-hydroxy metabolite. Soc. Neurosci. 17(1), 274.12, 1991.

94. D. D. Breimer. Variability in human drug metabolism and its implications. Int. J. Clin. Pharmacol. Res. III (6):399413, 1983.

95. L. M. Forrester, C. J. Henderson, M. J. Glancey, D. J. Back, B. K. Park, S. E. Ball, N. R. Kitteringham, A. W. McLaren, J. S. Miles, P. Skett, and C. R. Wolf. Relative expression of cytochrome P450 isoenzymes in human liver and association with the metabolism of drugs and xenobiotics. Biochem. J. 281:359368, 1992.

96. E. D. Kharasch and K. E. Thummel. Human alfentanil metabolism by cytochrome P450 3A3/4. An explanation for the interindividual variability in alfentanil clearance. Anesth. Analg. 76:10331039, 1993.

97. P. H. Beaune, P. G. Kremers, L. S. Kaminsky, J. De Graeve, A. Albert, and F. P. Guengerich. Comparison of monooxygenase activities and cytochrome P-450 isozyme concentrations in human liver microsomes. Drug Metab. Dispos. 14:437442, 1986.

98. F. P. Guengerich and C. G. Turvy. Comparison of levels of several human microsomal

cytochrome P-450 enzymes and epoxide hydrolase in normal and disease states using immunochemical analysis of surgical liver samples. J. Pharmacol. Exp. Ther. 256:11891194, 1991.

99. C. M. Hunt, W. R. Westerkam, and G. M. Stave. Effect of age and gender on the activity of human hepatic CYP3A. Biochem. Pharmacol. 44:275283, 1992.

100. D. L. Schmucker, K. W. Woodhouse, R. K. Wang, H. Wynne, O. F. James, M. McManus, and P. Kremers. Effects of age and gender on in vitro properties of human liver microsomal monooxygenases. Clin. Pharmacol. Ther. 48:365374, 1990.

101. C. Durnas, C. M. Loi, and B. J. Cusack. Hepatic drug metabolism and aging. Clin. Pharmacokinet. 19:359389, 1990.

102. W. Kalow. Genetic variation in the human hepatic cytochrome P-450 system. Eur. J. Clin. Pharmacol. 31:633641, 1987.

103. F. P. Guengerich. Polymorphism of cytochrome P-450 in humans. Trends Pharmacol. Sci. 10:107109, 1989.

104. M. V. Relling, J.-S. Lin, G. D. Ayers, and W. E. Evans. Racial and gender differences in N-acetyltransferase, xanthine oxidase, and CYP1A2 activities. Clin. Pharmacol. Ther. 52:643658, 1992.

105. S. Wanwimolruk, S. M. Wong, P. F. Coville, S. Viriyayudhakorn, and S. Thitiarchakul. Cigarette smoking enhances the elimination of quinine. Br. J. Clin. Pharmacol. 36:610614, 1993.

106. P. B. Watkins, D. K. Turgeon, S. K. Guthrie, A. L. Remen, and L. Grunhaus. P450IIIA (CYP3A) activity in severely depressed patients. J. Clin. Psychopharmacol. 13:366368, 1993.

107. S. Cholerton, A. K. Daly, and J. R. Idle. The role of individual human cytochromes P450 in drug metabolism and clinical response. Trends Pharmacol. Sci. 13:434439, 1992.

108. S. A. Wrighton and J. C. Stevens. The human hepatic cytochromes P450 involved in drug metabolism. Chem. Res. Toxicol. 22:121, 1992.

109. F. P. Guengerich. Characterization of human microsomal cytochrome P-450 enzymes. Annu. Rev. Pharmacol. Toxicol. 29:241264, 1989.

110. F. J. Gonzalez. Human cytochromes P450: problems and prospects. Trends Pharmacol. Sci. 13:346352, 1992.

111. W. Kalow and B.-K. Tang. The use of caffeine for enzyme assays: A critical appraisal. Clin. Pharmacol. Ther. 53:503514, 1993.

112. K. Lown, J. Kolars, K. Turgeon, R. Merion, S. A. Wrighton, and P. B. Watkins. The erythromycin breath test selectively measures P450IIIA in patients with severe liver disease. Clin. Pharmacol. Ther. 51:229238, 1992.

113. P. B. Watkins, D. K. Turgeon, P. Saenger, K. S. Lown, J. C. Kolars, T. Hamilton, K. Fishman, P. S. Guzelian, and J. J. Voorhees. Comparison of urinary 6-b-cortisol and the erythromycin breath test as measures of hepatic P450IIIA (CYP3A) activity. Clin. Pharmacol. Ther. 52:265273, 1992.

114. M. T. Kinirons, D. O'Shea, T. E. Downing, A. T. Fitzwilliam, L. Joellenbeck, J. D. Groopman, G. R. Wilkinson, and A. J. J. Wood. Absence of correlations among three putative in vivo probes of human cytochrome P4503A activity in young healthy men. Clin. Pharmacol. Ther. 54:621629, 1993.

115. B. K. Park and N. R. Kitteringham. Assessment of enzyme induction and enzyme inhibition in humans: Toxicological implications. Xenobiotica 20:11711185, 1990.

116. R. Zand, S. D. Nelson, J. T. Slattery, K. E. Thummel, T. F. Kalhorn, S. P. Adams, and J. M. Wright. Inhibition and induction of cytochrome P4502E1-catalyzed oxidation by isoniazid in humans. Clin. Pharmacol. Ther. 54:142149, 1993.

117. A. M. Baciewicz and F. A. Baciewicz, Jr. Ketoconazole and fluconazole drug interactions. Arch. Intern. Med. 153:19701976, 1993.

118. S. Pohjola-Sintonen, M. Viitasalo, L. Toivonen, and P. Neuvonen. Itraconazole prevents terfenadine metabolism and increases risk of torsades de pointes ventricular tachycardia. Eur. J. Clin. Pharmacol. 45:191193, 1993.

119. J. C. Kolars, P. Schmiedlin-Ren, J. D. Schuetz, C. Fang, and P. B. Watkins. Identification of rifampin-inducible P450IIIA4 (CYP3A4) in human small bowel enterocytes. J. Clin. Invest. 90:18711878, 1992.

120. H. Cheng, S. Schwartz, S. Vickers, J. D. Gilbert, R. D. Amin, B. Depuy, L. Liu, J. D. Rogers, S. M. Pond, C. A. Duncan, T. V. Olah, and W. F. Bayne. Metabolic disposition of simvastatin in patients with T-tube drainage. Drug Metab. Dispos. 22:139142, 1994.

121. R. F. Roberts and M. J. Fields. Monitoring radioactive compounds in high-performance liquid chromatographic eluates: Fraction collection versus on-line detection. J. Chromatogr. 342:2533, 1985.

122. H. Parvez, A. Reich, S. Lucas-Reich, and S. Parvez (Eds.) Progress in HPLC, Vol. 3, Flow Through Radioactivity Detection in HPLC. VSP BV, Utrecht, Netherlands, 1988.

123. R. D. McDowall. Sample preparation for biomedical analysis. J. Chromatogr. 492:358, 1989.

124. R. D. McDowall, J. C. Pearce, and G. S. Murkitt. Liquid-solid sample preparations in drug analysis. J. Pharm. Biomed. Anal. 4:321, 1986.

125. C. R. Blakley and M. L. Vestal. Thermospray interface for liquid chromatography/mass spectrometry. Anal. Chem. 55:750754, 1983.

126. M. L. Vestal and G. J. Fergusson. Thermospray liquid chromatography/mass spectrometer interface with direct electrical heating of the capillary. Anal. Chem. 57:23732378, 1985.

127. R. D. Voyksner and C. A. Haney. Optimization and application of thermospray high-performance liquid chromatography/mass spectrometry. Anal. Chem. 57:991996, 1985.

128. M. F. Bean, S. L. Pallante-Morell, D. M. Dulik, and C. Fenselau. Protocol for liquid chromatography/mass spectrometry of gluthathione conjugates using post-column solvent modification. Anal. Chem. 62:121124, 1990.

129. P. C. Winkler, D. D. Perkins, W. K. Williams, and R. F. Browner. Performance of an improved monodisperse aerosol generation interface for liquid chromatography/mass spectrometry. Anal. Chem. 60:489493, 1988.

130. R. C. Willoughby and R. F. Browner. Monodisperse aerosol generation interface for combining liquid chromatography with mass spectroscopy. Anal. Chem. 56:26262631, 1984.

131. T. D. Behymer, T. A. Bellar, and W. L. Budde. Liquid chromatography/particle beam/mass spectrometry of polar compounds of environmental interest. Anal. Chem. 62:16861690, 1990.

132. M. Barber, R. S. Bordoli, R. D. Sedgwick, and A. N. Tyler. Fast atom bombardment (F.A.B.): A new ion source for mass spectrometry. J. Chem. Soc. Chem. Comm. 325327, 1981.

133. R. M. Caprioli, W. T. Moore, B. DaGue, and M. Martin. Microbore high-performance

liquid chromatography-mass spectrometry for the analysis of proteolytic digests by continuous-flow fast bombardment mass spectrometry. J. Chromatogr. 443:355362, 1988.

134. M. A. Moseley, III, L. J. Deterding, J. S. M. de Wit, K. B. Tomer, R. T. Kennedy, N. Bragg, and J. W. Jorgenson. Optimization of a coaxial continuous flow fast atom bombardment interface between capillary liquid chromatography and magnetic sector mass spectrometry for the analysis of biomolecules. Anal. Chem. 61:15771584, 1989.

135. A. R. Woolfitt and C. A. J. Harbach. Liquid chromatography/mass spectrometry analysis of an enkephalin mixture using an Autospec dynamic liquid secondary-ion mass spectrometer interface. Rapid Commun. Mass Spectrom. 7:176178, 1993.

136. M. P. Balogh, C. C. Stacey, and K. M. Straub. Chromatographic developments in low flow delivery for liquid chromatography-mass spectrometry. J. Chromatogr. 11:267335, 1988.

137. K. M. Straub. Applications of fast atom bombardment and thermospray mass spectrometry to drug metabolism. Progress in Drug Metabolism (G. G. Gibson, ed.), Taylor and Francis, Ltd., Philadelphia, 1988, pp. 267335.

138. J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, and C. M. Whitehouse. Electrospray ionizationprinciples and practice. Mass Spectrom. Rev. 9:3770, 1990.

139. J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, and C. M. Whitehouse. Electrospray ionization for mass spectrometry of large biomolecules. Science 246:6471, 1989.

140. P. Kebarle and L. Tang. From ions in solution to ions in the gas phase: The mechanism of electrospray mass spectrometry. Anal. Chem. 65:972A986A, 1993.

141. M. G. Ikonomou, A. T. Blades, and P. Kebarle. Investigations of the electrospray interface for liquid chromatography/mass spectrometry. Anal. Chem. 62:957967, 1990.

142. M. G. Ikonomou, A. T. Blades, and P. Kebarle. Electrospray-ion spray: A comparison of mechanisms and performance. Anal. Chem. 63:19891998, 1989.

143. M. G. Ikonomou, A. T. Blades, and P. Kebarle. Electrospray mass spectrometry of methanol and water solutions suppression of electric discharge with SF6 gas. J. Am. Soc. Mass Spectrom. 2:497505, 1991.

144. I. Wampler, A. T. Blades, and P. Kebarle. Negative ion electrospray mass spectrometry of nucleotides: Ionization from water solution with SF6 discharge suppression. J. Am. Soc. Mass Spectrom. 4:289295, 1993.

145. S. A. Carr, M. E. Hemling, M. F. Bean, and G. D. Roberts. Integration of mass spectrometry in analytical biotechnology. Anal. Chem. 63:28022824, 1991.

146. S. A. McLuckey, G. J. Van Berkel, G. L. Glish, E. C. Huang, and J. D. Henion. Ion spray liquid chromatography/ion trap mass spectrometry determination of biomolecules. Anal. Chem. 63:375383, 1991.

147. T. G. Heath and A. B. Giordani. Reversed-phase capillary high-performance liquid chromatography with on-line UV, fluorescence and electrospray ionization mass spectrometric detection in the analysis of peptides and proteins. J. Chromatogr. 638:919, 1993.

148. P. A. Bottomley. Human in vivo NMR spectroscopy in diagnostic medicine: Clinical tool or research probe? Radiology 170:115, 1989.

149. M. E. Hemling, G. D. Roberts, W. Johnson, S. A. Carr, and T. R. Covey. Analysis of proteins and glycoproteins at the picomole level by on-line coupling of microbore high-

performance liquid chromatography with flow fast atom bombardment and electrospray mass spectrometry: A comparative evaluation. Biomed. Environ. Mass Spectrom. 19:677691, 1990.

150. R. D. Voyksner and T. Pack. Semiquantitative method for high-molecular-weight neuropeptides by high-performance liquid chromatography/thermospray mass spectrometry. Biomed. Environ. Mass Spectrom. 18:897903, 1989.

151. K. L. Duffin, T. Wachs, and J. D. Henion. Atmospheric pressure ion-sampling system for liquid chromatography/mass spectrometry analyses on a benchtop mass spectrometer. Anal. Chem. 64:6168, 1991.

152. M. A. Quilliam, B. A. Thompson, G. J. Scott, and K. W. M. Siu. Ion-spray mass spectrometry of marine neurotoxins. Rapid Commun. Mass Spectrom. 3:145150, 1989.

153. T. R. Covey, A. P. Bruins, and J. D. Henion. Comparison of thermospray and ion spray mass spectrometry in an atmospheric pressure ion source. Org. Mass Spectrom. 23:178186, 1988.

154. E. C. Huang, B. N. Pramanik, A. Tsarbopoulos, P. Reichert, A. K. Ganguly, P. P. Trotta, and T. L. Nagabhushan. Application of electrospray mass spectrometry in probing protein-protein and protein-ligand noncovalent interactions. J. Am. Soc. Mass Spectrom. 4:624630, 1993.

155. B. Ganem, Y.-T. Li, and J. D. Henion. Detection of noncovalent receptor-ligand complexes by mass spectrometry. J. Am. Chem. Soc. 113:62946296, 1991.

156. B. Ganem, Y.-T. Li, and J. D. Henion. Observation of noncovalent enzyme-substrate and enzyme-product complexes by ion-spray mass spectrometry. J. Am. Chem. Soc. 113:78187819, 1991.

157. A. P. Bruins, T. R. Covey, and J. D. Henion. Ion spray interface for combined liquid chromatography/atmospheric pressure ionization mass spectrometry. Anal. Chem. 59:26422646, 1987.

158. E. D. Lee, W. Mueck, J. D. Henion, and T. R. Covey. Liquid junction coupling for capillary zone electrophoresis/ion spray mass spectrometry. Biomed. Environ. Mass Spectrom. 18:844850, 1989.

159. E. Huang, J. D. Henion, and T. R. Covey. Packed-column supercritical fluid chromatography-mass spectrometry and supercritical fluid chromatography-tandem mass spectrometry with ionization at atmospheric pressure. J. Chromatogr. 511:257270, 1990.

160. J. J. Conboy, J. D. Henion, M. W. Martin, and J. A. Zweigenbaum. Ion chromatography/mass spectrometry for the determination of organic ammonium and sulfate compounds. Anal. Chem. 62:800807, 1990.

161. A. B. L. Lanting, A. P. Bruins, B. F. H. Drenth, K. de Jonge, K. Ensing, R. A. De Zeeuw, and D. K. F. Meijer. Identification with liquid chromatography-ionspray mass spectrometry of the metabolites of the enantiomers N-methyl dextrorphan and N-methyl levorphanol after rat liver perfusion. Biol. Mass Spectrom. 22:226234, 1993.

162. M. H. Allen and B. I. Shushan. Atmospheric pressure ionization-mass spectrometry detection for liquid chromatography and capillary electrophoresis. LC/GC 11:112126, 1993.

163. B. Ganem and J. D. Henion. Detecting noncovalent complexes of biological macromolecules: New applications of ion-spray mass spectrometry. Chemtracts-Org. Chem. 6:122, 1993.

164. J. W. Jorgenson and K. D. Lukacs. Capillary zone electrophoresis. Science 222:266272, 1983.

165. W. M. A. Niessen and H. Poppe. Open-tubular liquid chromatography-mass spectrometry using direct liquid introduction. J. Chromatogr. 323:3746, 1985.

166. J. S. M. deWit, L. J. Deterding, M. A. Moseley, III, K. B. Tomer, and J. W. Jorgenson. Design of a coaxial continuous flow fast atom bombardment probe. Rapid Commun. Mass Spectrom. 2:100104, 1988.

167. M. J.-F. Suter and R. M. Caprioli. An integral probe for capillary zone electrophoresis/continuous-flow fast atom bombardment mass spectrometry. J. Am. Soc. Mass Spectrom. 3:198206, 1992.

168. W. M. A. Niessen, U. R. Tjaden, and J. van der Greef. Capillary electrophoresis-mass spectrometry. J. Chromatogr. 636:319, 1993.

169. C. E. Parker, J. R. Perkins, K. B. Tomer, Y. Shida, K. O'Hara, and M. Kono. Application of nanoscale packed capillary liquid chromatography (75 micrometer

id) and capillary zone electrophoresis/electrospray ionization mass spectrometry to the analysis of macrolide antibiotics. J. Am. Soc. Mass Spectrom. 3:563574, 1992.

170. F. Hillenkamp, M. Karas, R. C. Beavis, and B. T. Chait. Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. Anal. Chem. 63:1193A1203A, 1991.

171. M. Karas, D. Bachmann, and F. Hillenkamp. Influence of the wavelength in high-irradiance ultraviolet laser desorption mass spectrometry of organic molecules. Anal. Chem. 57:29352939, 1985.

172. M. Karas, D. Bachman, U. Bahr, and F. Hillenkamp. Matrix-assisted ultraviolet laser desorption of non-volatile compounds. Int. J. Mass Spectrom. Ion Proc. 78:5368, 1987.

173. F. Hillenkamp and M. Karas. Mass spectrometry of peptides and proteins by matrix-assisted ultraviolet laser desorption/ionization. Methods in Enzymology (J. A. McCloskey, ed.), Academic Press, San Diego, 1990, pp. 280295.

174. M. Karas, U. Bahr, A. Ingendoh, and F. Hillenkamp. Laser desorption/ionization mass spectrometry of proteins of mass 100,000 to 250,000 dalton. Angew. Chem. Intl. Ed. Engl. 28:760761, 1989.

175. R. A. Yost and R. K. Boyd. Tandem mass spectrometry: quadrupola and hybrid instruments. Methods in Enzymology (J. A. McCloskey, ed.), Academic Press, San Diego, 1990, pp. 154200.

176. M. L. Gross. Tandem mass spectrometry: Multisector magnetic instruments. Methods in Enzymology (J. A. McCloskey, ed.), Academic Press, San Diego, 1990, pp. 131153.

177. G. L. Glish and S. A. McLuckey. Analyzer scan modes for hybrid mass spectrometers. Org. Mass Spectrom. 24:470478, 1989.

178. R. J. Perchalski, R. A. Yost, and B. J. Wilder. Structural elucidation of drug metabolites by triple-quadrupole mass spectrometry. Anal. Chem. 54:14661471, 1982.

179. R. A. Yost, R. J. Perchalski, H. O. Brotherton, J. V. Johnson, and M. B. Budd. Pharmaceutical and clinical analysis by tandem mass spectrometry. Talanta 31:929935, 1984.

180. P. E. Haroldsen, M. H. Reilly, H. Hughes, S. J. Gaskell, and C. J. Porter. Characterization of glutathione conjugates by fast atom bombardment/tandem mass spectrometry. Biomed. Environ. Mass Spectrom. 15:615621, 1988.

181. K. D. Ballard, M. J. Raftery, H. Jaeschke, and S. J. Gaskell. Multiple scan modes in

the hybrid tandem mass spectrometric screening and characterization of the glutathione conjugate of 2-furamide. J. Am. Soc. Mass Spectrom. 2:5568, 1991.

182. M. Leal, M. J. Hayes, and M. L. Powell. The metabolism of CGS 15873 in man using stable isotope pattern recognition techniques. Biopharm. Drug Dispos., 13, 617628 (1992).

183. T. A. Baillie. Advances in the application of mass spectrometry to studies of drug metabolites, pharmacokinetics and toxicology. Int. J. Mass Spectrom. Ion Processes 118119:289314, 1992.

184. J. K. Nicholson and I. D. Wilson. High resolution nuclear magnetic resonance spectroscopy of biological samples as an aid to drug development. Prog. Drug Res. 31:427479, 1987.

185. M. J. W. Prior, R. J. Maxwll, and J. R. Griffiths. Fluorine-19 NMR spectroscopy and imaging in-vivo. In NMR Basic Principles and Progress. Edited by P. Diehl,

E. Fluck, H. Gunther, R. Kosfeld, and J. Seelig. Springer-Verlag, Berlin, 1992, pp. 101130.

186. M. C. Malet-Martino and R. Martino. Magnetic resonance spectroscopy: A powerful tool for drug metabolism studies. Biochimie 74:785800, 1992.

187. A. E. Derome. Modern NMR Techniques for Chemistry Research. Pergamon Press, New York, 1987.

188. R. W. Kriwacki and T. P. Pitner. Current aspects of practical two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy: Applications to structure elucidation. Pharm. Res. 6:531554, 1989.

189. J. R. Everett. Biofluid NMR spectroscopy in the study of the metabolism of b-lactam antibiotics. In Bioanalytical Approaches for Drugs, Including Anti-Asthmatics and Metabolites, Vol. 22. Edited by E. Reid and I.D. Wilson. Royal Society of Chemistry, Cambridge, 1992, pp. 310.

190. M. C. Malet-Martino and R. Martino. The application of nuclear magnetic resonance spectroscopy to drug metabolism studies. Xenobiotica 19:583607, 1989.

191. P. J. D. Foxall, J. A. Parkinson, I. H. Sadler, J. C. Lindon, and J. K. Nicholson. Analysis of biological fluids using 600 MHz proton NMR spectroscopy: Application of homonuclear two-dimensional J-resolved spectroscopy to urine and blood plasma for spectral simplification and assignment. J. Pharm. Biomed. Anal. 11:2131, 1993.

192. P. J. D. Foxall, M. Spraul, R. D. Farrant, L. C. Lindon, G. H. Neild, and J. K. Nicholson. 750 MHz proton-nmr spectroscopy of human blood plasma. J. Pharm. Biomed. Anal. 11:267276, 1993.

193. J. K. Nicholson and I. D. Wilson. High resolution proton magnetic resonance spectroscopy of biological fluids. Prog. NMR Spec. 21:449501, 1989.

194. D. L. Rabenstein and S. Fan. Proton nuclear magnetic resonance spectroscopy of aqueous solutions: Complete elimination of the water resonance by spin-spin relaxation. Anal. Chem. 58:31783184, 1986.

195. I. D. Wilson and J. K. Nicholson. Solid phase extraction chromatography and NMR spectroscopy (SPEC-NMR) for the rapid identification of drug metabolites in urine. J. Pharm. Biomed. Anal. 6:151165, 1988.

196. T. F. Woolf, S. M. Bjorge, A. Black, and T. Chang. Metabolism of the acyl-CoA: cholesterol acyltransferase (ACAT) inhibitor, 2,2-dimethyl-N-(2,4,6-

trimethoxyphenyl)dodecanamide, CI-976, in rat and monkey: omega-/beta-oxidation pathway. Drug Metab. Dispos. 19:696702, 1991.

197. M. Spraul, M. Hofmann, P. Dvortsak, J. K. Nicholson, and I. D. Wilson. Liquid chromatography coupled with high-field proton NMR for profiling human urine for endogenous compounds and drug metabolites. J. Pharm. Biomed. Anal. 10:601605, 1992.

198. M. Spraul, M. Hofmann, P. Dvortsak, J. K. Nicholson, and I. D. Wilson. High-performance liquid chromatography coupled to high-field proton nuclear magnetic resonance spectroscopy: Application to the urinary metabolites of ibuprofen. Anal. Chem. 65:327330, 1993.

10
Hepatic Microsomes and Heterologous Expression Systems as In Vitro Models for Human Drug Metabolism

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I. Introduction

The aim in pharmaceutical drug discovery and development is the identification of a safe and potent drug for human use. Safety is based on experiments in which animals are used to predict the risk for the application in humans. At an early point of development, few experimental approaches are available to ensure

that the species chosen for preclinical work is similar to humans. This similarity should ideally be achieved with respect to the quantitative (pharmacokinetic) and qualitative (metabolism) changes of the test substance. Human hepatic microsomes and drug-metabolizing enzymes expressed in a heterologous system can be used to predict the metabolism in humans and to establish the relevance of the species used in toxicological investigations. Later in the development of a drug, the structures of the metabolites will be elucidated. Because in vitro systems can produce metabolites in a comparatively simple biological matrix, these systems can be applied to produce amounts of metabolites sufficient for structure elucidation.

Human drug-metabolizing enzymes, especially those of the cytochrome P450 (CYP) family, are present in human liver microsomes or human hepatocytes or can be expressed from their cDNAs in a foreign cell that normally does not express CYP enzymes. This heterologous expression has become a widely used tool to characterize the metabolic pathways of numerous drugs.

II. Human Liver Microsomes

Human liver tissue, which can be obtained from kidney transplant donors or biopsies, can be stored at -80°C over a long period of time without an apparent loss of catalytic activity of many drug-metabolizing enzymes. Microsomes prepared from these livers provide a system that allows for the determination of numerous metabolic reactions prior to the first application of the drug to humans in vivo. Microsomal fractions are easy to prepare and can also be stored without a major loss of catalytic activity. This allows for the establishment of tissue banks that can be characterized on the functional, protein, RNA, or DNA level for the expression of enzymes or their mRNAs and for the presence of genetic polymorphisms of drug oxidation in these samples. Table 1 summarizes several studies in which human liver microsomes have been employed either to characterize the specificity of certain CYP isozymes or to elucidate the role of these isozymes in the metabolism of a certain compound. Although the latest update on the CYP superfamily lists 221 cytochrome P450 genes and 12 putative pseudogenes [1] that have been characterized in 31 eukaryotes and 11 prokaryotes, 12 families exist in all mammals, including humans. This striking multiplicity is in contrast to the investigations of only a limited number of CYP isozymes in human liver microsomes, as seen in Table 1. Most of these studies are concerned with CYP1A, 2C, 2D6, 2E1, or 3A.

Most of our knowledge about the involvement of distinct forms of cytochromes P450 in the metabolism of drugs and carcinogens in humans is based on investigations with human liver microsomes. CYP enzymes can be expressed in heterologous systems (see below) and show a certain activity for a given drug. It is, however, not clear how

important the contribution of the expressed enzyme is in vivo. Only the comparison to the complex situation in the mixture

Table 1 Studies with Human Liver Microsomes

Compound	CYP Investigated	Remarks	References
	1A2, 2C, 2E1, 3A	100 liver samples compared; disease states	2
1-Nitropyrene	3A4		3
4-Nitrophenol	2E1		4
4-Nitrophenol	2E1		5
6-Aminochrysene	2B6	High interindividual variation in 50 human livers	6
Alkoxyresorufins	1A2, 2A6, 3A4, 2C	Species comparison	7
Amiodarone	3A		8
Bufuralol	2D6	2D6 Marker	916
Cannabinoids	2C		17
Cinnarizine	2D6		18
Citalopram	2D6		19
Clozapine	2D6		20
Cocaine	3A		21
Cortisol	3A	CYP isoforms	22
Coumarin	2A3		2326
CQA 206291	3A4		27
Cyclophosphamide	2B	Metabolic activation	28
Cyclosporin	3A		27,2939
Debrisoquine	2D6	2D6 polymorphism = debrisoquine polymorphism	11,12,16,4045
Desipramine	2D6		46
Dextromethorphan	2D6	2D6 Marker	10,12,20,4762
Epipodophyllotoxins	3A4		30
Erythromycin	3A	CYP isoforms 3A Marker	22
Estradiol	3A		63
FK506	3A		64,65
Flunarizine	2D6		18
Fluoxetine	2D6		19
Fluvoxamine	2D6		19
Hydrocodone	2D6		49
Imipramine	2D6, 1A2		6668
Iphosphamide	3A	Metabolic activation	28
Ketoconazole	3A		6668
Lidocaine	2D6, 3A		6971
Lovastatin	3A		72

Mephenytoin	2C	S-Mephenytoin-4-hydroxylase Polymorphism	4,43,67,7394

Table 1 Continued

Compound	CYP Investigated	Remarks	References
Mexiletine	2D6		42,52,55,95
Midazolam	3A		27,29,30,48,9698
Minaprine	2D6		41
Nifedipine	3A		3,8,9,26,64,99104
Norcocaine	3A4		105
Omeprazole	2C, 3A, 2D6		74,106
Paroxetine	2D6		40
Paroxetine	2D6		19
Phenytoin	2C		107,108
Quinolones	1A2		109
Sertaline	2D6		19
Sparteine	2D6	Human and monkey	110
Tacrine	1A2		111
Tamoxifen	2D6	Not 2D6 related	112
Taxol	2C, 3A4		113
Terbinafine	3A		35
Theophylline	1A2, 3A3, 2E1		114
Tienilic acid	2C		76,115,116
Tolbutamide	2C		9,27,35,79,82,108,117,118
Triazolam	3A		98
Tropisetron	2D6		47
Verapamil		In vivo prediction from in vitro kinetics	119
Warfarin			120123
Zonisamide	3A	Correlation in 21 human livers	124

of CYP isozymes in human liver can establish the importance of an individual cytochrome P450 for overall metabolism.

III. Heterologous Expression of P450 Enzymes in Mammalian Cells

A. Transient Expression

1. COS cells

COS-1 cells [27,77,125146] are kidney cells derived from an African green monkey cell line, CV-1, which has been transformed to express stably the large T antigen of the SV40

virus [125]. Large T antigen is a transcription factor that

is necessary to transcribe efficiently plasmids that carry the SV40 origin of replication. These plasmids are readily available and cDNAs for P450 isozymes can be inserted and expressed [132]. When these plasmids are transfected into COS-1 cells, the plasmid is replicated, the DNA is transcribed into mRNA, which is then translated into the polypeptide chain. To this apoprotein, the prosthetic heme group is covalently attached and the resulting holoenzyme is inserted into the membrane of the endoplasmic reticulum, so that it can functionally couple with the constitutively expressed NADPH-cytochrome P450 reductase. This reductase-cytochrome P450 complex is functionally complete and reactions are driven by the endogenous NADPH. As in most of the other heterologous expression systems, endogenous cytochromes P450 are not detected and, therefore, the P450 isozyme of interest can be investigated as an isolated entity. Since the compounds whose metabolism is to be investigated are mostly hydrophobic, they can cross the plasma membrane and can subsequently be metabolized by the expressed P450 enzyme. Metabolites will in most cases also be hydrophobic enough to diffuse out of the cell. This allows for the detection of metabolites in the cell culture medium. Reviews of the expression of CYP enzymes in COS cells have been published elsewhere [137,139].

The COS cell system has been exploited in many ways. Zuber et al. were the first to use the COS-1 system for the heterologous expression of cytochrome P450 enzymes [127]. They demonstrated that cytochrome $P450_{17a}$ (CYP17 a) had both intrinsic 17a-hydroxylase and 17,20-lyase activity for pregnenolone. Subsequently, many other groups have applied this system for the identification of members of the steroid-metabolizing enzyme systems [127,129,134136,138,144,150,151]. Those and other studies were used to identify, confirm, or assign specific enzymatic activities to cloned CYP cDNAs. Other investigators have then used this system to identify the critical role of CYP enzymes in the metabolism of drugs and carcinogens [27,77,142,147,154].

The COS cell system can be employed universally for the heterologous expression of many forms of CYP enzymes (see Table 2). However, the handling requires some knowledge of basic molecular biological techniques. Because the achieved expression is transient, a new transfection has to be performed for each experiment.

2. Vaccinia Virus-Directed Expression

The infection of hepatoma cells with vaccinia derived viruses can be used to express CYP enzymes [155165]. This expression system is reviewed in detail by Gonzalez et al. [162]. Handling of the large genome of the virus requires transfer vectors and the resulting high specific content of functional protein is an advantage of this system. However, the infectivity of the viruses requires the application of this system only by laboratories with biosafety level 2 standards.

Table 2 Cytochromes P450 Expressed in COS Cells

CYP Investigated	References
1A2	134,147
2C1	148
2C3	148
2C4	148,149
2C5	136,148,149
2C9	77,142
2C18	77
2C19	77
3A4	27
3A5	27
7	150
11A1, 17	132
11B	151
11B family	135,138,141,144,145
17	133
17	127
19	152
21A	153

B. Stably Transformed Cell Lines

1. AHH-1 Cells

Human cell lines derived from AHH-1 TK +/-human lymphoblasts express low amounts of cytochrome P450 (CYP1A1) constitutively [28,166178] and can be genetically engineered to express other CYP enzymes or epoxide hydrolase. Using extrachromosomal vectors based on constructs that contain OriP sequences derived from Epstein-Barr virus [28,169,170,172177], CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C6, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 have been expressed in this model (for review see [176]). The yields of functionally expressed protein vary and seem to depend not only on the DNA construct but also on the protein to be expressed. The efficient expressors CYP1A2, CYP2A6, and CYP2D6 are expressed at the ~30 pmol/mg microsomal protein level, which compares favorably with the estimated levels of individual CYP isozymes in human liver microsomes, of ~1050 pmol/mg. The low expressors CYP2E1 [178] and CYP3A4 [172], however, are expressed to only ~1 pmol/mg protein. The human lymphoblast cell lines have also been used to express 5 CYP cDNAs simultaneously at the 1.53 pmol/mg protein level. Although the primary use of the resulting MCL-5 cells could be as a screening system for promutagen activation, they should also be applicable to the predic-

tion of possible metabolites in humans. An interesting application is the use of transformed AHH cells to detect active metabolites of anticancer drugs [28] that inhibit the proliferation of transfected cells but not that of control cells.

The commercial availability of both the cell lines and microsomal fractions of these cells has enabled the identification of critical enzymes in the metabolism of new chemical entities.

2. V79 Cells.

V79 Chinese hamster cells can be genetically engineered to express cytochrome P450 enzymes. Although it is possible to use these cells to identify critical P450 isozymes for the metabolism of drugs [47,179188], most of the applications for this model system are in toxicology since untransformed V79 cells have long been used in mutagenicity and cytotoxicity testing.

IV. Heterologous Expression of P450 Enzymes in Insect Cells: Bacculovirus

Bacculovirus includes a family of viruses that infect only insect cells [154,189194]. Thus, these viruses are safer to handle than the generally infective vaccinia virus. Bacculovirus has been used to design an eukaryotic system with the highest level for protein expression. The reported levels of expression range from 1 to 500 mg/L infected cells. CYP enzymes can also be expressed in this system; however, the employed insect cells show a deficiency in the incorporation of heme into the apoprotein. This problem can at least partly be corrected by the addition of hemin to the culture medium. Using this technique, Gonzalez et al. have achieved an expression of up to 0.7 nmol/mg total cell lysate protein of CYP holoenzyme [191].

V. Heterologous Expression in Yeast

Table 3 gives a comprehensive overview over the application of yeast in the expression of CYP enzymes. Yeast has the potential to synthesize large amounts of functional protein. The application of yeast for the investigation of drug metabolism has been the subject of a number of reviews [195200]. Expression levels of 8 nmol P450/L have been obtained [195].

In general, human liver microsomes and heterologous expression systems have been used to identify, confirm, or assign specific enzymatic activities to cloned CYP cDNAs. This allows for the comparison of human metabolic reactions with those of animals in an early phase of drug development. Therefore, these studies should contribute to an increase in the predictability of animal experiments for application to humans.

Table 3 Expression of CYPs in Yeast

CYP Investigated	Reference(s)
1A	201217
2A	218
2B	219221
2C	73,80,85,217,222229
17	230,231
4A	232
3A4	216,217,233236
2E1	237
2F	238
2D6	239,240

References

1. D. R. Nelson, T. Kamataki, D. J. Waxman, F. P. Guengerich, R. W. Estabrook, R. Feyereisen, F. J. Gonzalez, M. J. Coon, I. C. Gunsalus, O. Gotoh, K. Okuda, and D. W. Nebert. The P450 superfamily: Update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. DNA Cell Biol. 12:151, 1993.

2. F. P. Guengerich and C. G. Turvey. Comparison of levels of several human microsomal cytochrome P-450 enzymes and epoxide hydrolase in normal and disease states using immunochemical analysis of surgical liver samples. J. Pharmacol. Exp. Ther. 256:11891194, 1991.

3. K. J. Silvers, T. Chazinski, M. E. McManus, S. L. Bauer, F. J. Gonzalez, H. V. Gelboin, P. Maurel, and P. C. Howard. Cytochrome P-450 3A4 (nifedipine oxidase) is responsible for the C-oxidative metabolism of 1-nitropyrene in human liver microsomal samples. Cancer Res. 52:62376243, 1992.

4. W. Tassaneeyakul, M. W. Veronese, D. J. Birkett, F. J. Gonzalez, and J. O. Miners. Validation of 4-nitrophenol as an in vitro substrate probe for human liver CYP2E1 using cDNA expression and microsomal kinetic techniques. Biochem. Pharmacol. 46:19751981, 1993.

5. W. Tassaneeyakul, M. E. Veronese, D. J. Birkett, and J. O. Miners. Highperformance liquid chromatographic assay for 4-nitrophenol hydroxylation, a putative cytochrome P-4502E1 activity, in human liver microsomes. J. Chromatogr. Biomed. Appl. 616:7378, 1993.

6. M. Mimura, T. Baba, H. Yamazaki, S. Ohmori, Y. Inui, F. J. Gonzalez, F. P. Guengerich, and T. Shimada. Characterization of cytochrome P-450 2B6 in human liver microsomes.

Drug Metab. Dispos. 21:10481056, 1993.

7. R. J. Weaver, S. Thompson, G. Smith, M. Dickins, C. R. Elcombe, R. T. Mayer, and M. D. Burke. A comparative study of constitutive and induced alkoxyresorufin O-dealkylation and individual cytochrome P450 forms in cynomolgus monkey

(*Macaca fascicularis*), human, mouse, rat and hamster liver microsomes. Biochem. Pharmacol. 47:763773, 1994.

8. G. Fabre, B. Julian, B. Saint-Aubert, H. Joyeux, and Y. Berger. Evidence for CYP3A-mediated N-deethylation of amiodarone in human liver microsomal fractions. Drug Metab. Dispos. 21:978985, 1993.

9. R. G. Knodell, D. G. Browne, G. P. Gwozdz, W. R. Brian, and F. P. Guengerich. Differential inhibition of individual human liver cytochromes P-450 by cimetidine. Gastroenterology 101:16801691, 1991.

10. T. Kronbach. Bufuralol, dextromethorphan, and debrisoquine as prototype substrates for human P450IID6. Methods Enzymol. 206:509517, 1991.

11. U. M. Zanger, F. Vilbois, J. P. Hardwick, and U. A. Meyer. Absence of hepatic cytochrome P450bufl causes genetically deficient debrisoquine oxidation in man. Biochemistry 27:54475454, 1988.

12. T. Kronbach, D. Mathys, J. Gut, T. Catin, and U. A. Meyer. High-performance liquid chromatographic assays for bufuralol 1'-hydroxylase, debrisoquine 4-hydroxylase, and dextromethorphan O-demethylase in microsomes and purified cytochrome P-450 isozymes of human liver. Anal. Biochem. 162:2432, 1987.

13. P. Dayer, T. Leemann, A. Küpfer, T. Kronbach, and U. A. Meyer. Stereo- and regioselectivity of hepatic oxidation in man -Effect of the debrisoquine/sparteine phenotype on bufuralol hydroxylation. Eur. J. Clin. Pharmacol. 31:313318, 1986.

14. J. Gut, T. Catin, P. Dayer, T. Kronbach, U. Zanger, and U. A. Meyer. Debrisoquine/sparteine-type polymorphism of drug oxidation. Purification and characterization of two functionally different human liver cytochrome P-450 isozymes involved in impaired hydroxylation of the prototype substrate bufuralol. J. Biol. Chem. 261:1173411743, 1986.

15. J. Gut, R. Gasser, P. Dayer, T. Kronbach, T. Catin, and U. A. Meyer. Debrisoquine-type polymorphism of drug oxidation: Purification from human liver of a cytochrome P-450 isozyme with high activity for bufuralol hydroxylation. FEBS Lett. 173:287290, 1984.

16. M. E. McManus, A. R. Boobis, R. F. Minchin, D. M. Schwartz, S. Murray, D. S. Davies, and S. S. Thorgeirsson. Relationship between oxidation metabolism of 2-acetylaminofluorene debrisoquine, bufuralol, and aldrin in human liver microsomes. Cancer Res. 44:56925697, 1984.

17. L. M. Bornheim and M. A. Correia. Purification and characterization of the major

hepatic cannabinoid hydroxylase in the mouse: A possible member of the cytochrome P-450IIC subfamily. Mol. Pharmacol. 40:228234, 1991.

18. S. Narimatsu, S. Kariya, S. Isozaki, S. Ohmori, M. Kitada, S. Hosokawa, Y. Masubuchi, and T. Suzuki. Involvement of CYP2D6 in oxidative metabolism of cinnarizine and flunarizine in human liver microsomes. Biochem. Biophys. Res. Commun. 193:12621268, 1993.

19. H. K. Crewe, M. S. Lennard, G. T. Tucker, F. R. Woods, and R. E. Haddock. The effect of selective serotonin re-uptake inhibitors on cytochrome P4502D6 (CYP2D6) activity in human liver microsomes. Br. J. Clin. Pharmacol. 34:262265, 1992.

20. V. Fischer, B. Vogels, G. Maurer, and R. E. Tynes. The antipsychotic clozapine is metabolized by the polymorphic human microsomal and recombinant cytochrome P450 2D6. J. Pharmacol. Exp. Ther. 260:13551360, 1992.

21. P. Pellinen, P. Honkakoski, F. Stenbäck, M. Niemitz, E. Alhava, O. Pelkonen, M. A. Lang, and M. Pasanen. Cocaine N-demethylation and the metabolism-related hepatotoxicity can be prevented by cytochrome P450 3A inhibitors. Eur. J. Pharmacol. Environ. Toxicol. Pharmacol. 270:3543, 1994.

22. C. M. Hunt, P. B. Watkins, P. Saenger, G. M. Stave, N. Barlascini, C. O. Watlington, J. T. Wright, Jr., and P. S. Guzelian. Heterogeneity of CYP3A isoforms metabolizing erythromycin and cortisol. Clin. Pharmacol. Ther. 51:1823, 1992.

23. S. Yamano, J. Tatsuno, and F. J. Gonzalez. The CYP2A3 gene product catalyzes coumarin 7-hydroxylation in human liver microsomes. Biochemistry 29:13221329, 1990.

24. J. S. Miles, A. W. McLaren, L. M. Forrester, M. J. Glancey, M. A. Lang, and C. R. Wolf. Identification of the human liver cytochrome P-450 responsible for coumarin 7-hydroxylase activity. Biochem. J. 267:365371, 1990.

25. C.-H. Yun, T. Shimada, and F. P. Guengerich. Purification and characterization of human liver microsomal cytochrome P-450 2A6. Mol. Pharmacol. 40:679685, 1991.

26. I. Dalet-Beluche, X. Boulenc, G. Fabre, P. Maurel, and C. Bonfils. Purification of two cytochrome P450 isozymes related to CYP2A and CYP3A gene families from monkey (baboon, *Papio papio*) liver microsomescross reactivity with human forms. Eur. J. Biochem. 204:641648, 1992.

27. S. E. Ball, G. Maurer, M. Zollinger, M. Ladona, and A. E. M. Vickers. Characterization of the cytochrome P-450 gene family responsible for the N-dealkylation of the ergot alkaloid CQA 206291 in humans. Drug Metab. Dispos. 20:5663, 1992.

28. T. K. H. Chang, G. F. Weber, C. L. Crespi, and D. J. Waxman. Differential activation of cyclophosphamide and ifosphamide by cytochromes P-450 2B and 3A in human liver microsomes. Cancer Res. 53:56295637, 1993.

29. G. N. Kumar, U. K. Walle, and T. Walle. Cytochrome P450 3A-mediated human liver microsomal taxol 6a-hydroxylation. J. Pharmacol. Exp. Ther. 268:11601165, 1994.

30. M. V. Relling, J. Nemec, E. G. Schuetz, F. J. Gonzalez, and K. R. Korzekwa. O-demethylation of epipodophyllotoxins is catalyzed by human cytochrome P450 3A4. Mol. Pharmacol. 45:352358, 1994.

31. L. Pichard, I. Fabre, M. Daujat, J. Domergue, H. Joyeux, and P. Maurel. Effect of corticosteroids on the expression of cytochromes P450 and on cyclosporin A oxidase activity in primary cultures of human hepatocytes. Mol. Pharmacol. 41:10471055, 1992.

32. A. E. M. Vickers, V. Fischer, S. Connors, R. L. Fisher, J.-P. Baldeck, G. Maurer, and K. Brendel. Cyclosporin A metabolism in human liver, kidney, and intestine slices: Comparison to rat and dog slices and human cell lines. Drug Metab. Dispos. 20:802809, 1992.

33. L. Pichard, I. Fabre, G. Fabre, J. Domergue, B. S. Aubert, G. Mourad, and P. Maurel. Cyclosporin A drug interactions. Screening for inducers and inhibitors of

cytochrome P-450 (cyclosporin A oxidase) in primary cultures of human hepatocytes and in liver microsomes. Drug Metab. Dispos. 18:595606, 1990.

34. T. Aoyama, S. Yamano, D. J. Waxman, D. P. Lapenson, U. A. Meyer, V. Fischer, R. Tyndale, T. Inaba, W. Kalow, H. V. Gelboin, and F. J. Gonzalez. Cytochrome P-450 hPCN3, a novel cytochrome P-450 IIIA gene product that is differentially expressed in adult human liver. cDNA and deduced amino acid sequence and distinct specificities of cDNA-expressed hPCN1 and hPCN3 for the metabolism of steroid hormones and cyclosporine. J. Biol. Chem. 264:1038810395, 1989.

35. D. J. Back, P. Stevenson, and J. F. Tjia. Comparative effects of two antimycotic agents, ketoconazole and terbinafine on the metabolism of tolbutamide, ethinyloestradiol, cyclosporin and ethocycoumarin by human liver microsomes in vitro. Br. J. Clin. Pharmacol. 28:166170, 1989.

36. J. Combalbert, I. Fabre, G. Fabre, I. Dalet, J. Derancourt, J. P. Cano, and P. Maurel. Metabolism of cyclosporin A. IV. Purification and identification of the rifampicin-inducible human liver cytochrome P-450 (cyclosporin A oxidase) as a product of P450IIIA gene subfamily. Drug Metab. Dispos. 17:197207, 1989.

37. P. M. Shaw, T. S. Barnes, D. Cameron, J. Engeset, W. T. Melvin, G. Omar, J. C. Petrie, W. R. Rush, C. P. Snyder, P. H. Whiting, C. R. Wolf, and M. D. Burke. Purification and characterization of an anticonvulsant-induced human cytochrome P-450 catalysing cyclosporin metabolism. Biochem. J. 263:653663, 1989.

38. J. F. Tjia, D. J. Back, and A. M. Breckenridge. Calcium channel antagonists and cyclosporine metabolism: In vitro studies with human liver microsomes. Br. J. Clin. Pharmacol. 28:362365, 1989.

39. T. Kronbach, V. Fischer, and U. A. Meyer. Cyclosporine metabolism in human liver: Identification of a cytochrome P-450III gene family as the major cyclosporine-metabolizing enzyme explains interactions of cyclosporine with other drugs. Clin. Pharmacol. Ther. 43:630635, 1988.

40. J. C. Bloomer, F. R. Woods, R. E. Haddock, M. S. Lennard, and G. T. Tucker. The role of cytochrome P4502D6 in the metabolism of paroxetine by human liver microsomes. Br. J. Clin. Pharmacol. 33:521523, 1992.

41. F. Marre, G. Fabre, B. Larcarelle, M. Bourrie, J. Catalin, Y. Berger, R. Rahmani, and J.-P. Cano. Involvement of the cytochrome P-450IID subfamily in minaprine 4-hydroxylation by human hepatic microsomes. Drug Metab. Dispos. 20:316321, 1992.

42. J. Turgeon, C. Fiset, R. Giguère, M. Gilbert, K. Moerike, J. R. Rouleau, H. K. Kroemer, M. Eichelbaum, O. Grech-Bélanger, and P. M. Bélanger. Influence of debrisoquine phenotype and of quinidine on mexiletine disposition in man. J. Pharmacol. Exp. Ther. 259:789798, 1991.

43. M. V. Relling, W. E. Evans, R. Fonné-Pfister, and U. A. Meyer. Anticancer drugs as inhibitors of two polymorphic cytochrome P450 enzymes, debrisoquin and mephenytoin hydroxylase, in human liver microsomes. Cancer Res. 49:6871, 1989.

44. C. Birgersson, A. Blanck, K. Woodhouse, B. Mellstrom, and C. von Bahr. Comparative metabolism of debrisoquine, 7-ethoxyresorufin and benzo(a)pyrene in liver microsomes from humans, and from rats treated with cytochrome P-450 inducers. Acta Pharmacol. Toxicol. 57:117120, 1985.

45. E. Spina, C. Birgersson, C. von Bahr, O. Ericsson, B. Mellstrom, E. Steiner, and F. Sjoqvist. Phenotypic consistency in hydroxylation of desmethylimipramine and debrisoquine in healthy subjects and in human liver microsomes. Clin. Pharmacol. Ther. 36:677682, 1984.

46. K. Brosen, J. G. Hansen, K. K. Nielsen, S. H. Sindrup, and L. F. Gram. Inhibition by paroxetine of desipramine metabolism in extensive but not in poor metabolizers of sparteine. Eur. J. Clin. Pharmacol. 44:349355, 1993.

47. V. Fischer, A. E. M. Vickers, F. Heitz, S. Mahadevan, J.-P. Baldeck, P. Minery, and R. Tynes. The polymorphic cytochrome P-4502D6 is involved in the metabolism of both 5-hydroxytryptamine antagonists, tropisetron and ondansetron. Drug Metab. Dispos. 22:269274, 1994.

48. T. Leemann, P. Bonnabry, and P. Dayer. Selective inhibition of major drug metabolizing cytochrome P450 isozymes in human liver microsomes by carbon monoxide. Life Sci. 54:951956, 1994.

49. S. V. Otton, M. Schadel, S. W. Cheung, H. L. Kaplan, U. E. Busto, and E. M. Sellers. CYP2D6 phenotype determines the metabolic conversion of hydrocodone to hydromorphone. Clin. Pharmacol. Ther. 54:463472, 1993.

50. D. Wu, S. V. Otton, B. A. Sproule, U. Busto, T. Inaba, W. Kalow, and E. M. Sellers. Inhibition of human cytochrome P450 2D6 (CYP2D6) by methadone. Br. J. Clin. Pharmacol. 35:3034, 1993.

51. L. Koymans, N. P. E. Vermeulen, S. A. B. E. Van Acker, J. M. Te Koppele, J. J. P. Heykants, K. Lavrijsen, W. Meuldermans, and G. M. Donné-Op den Kelder. A predictive model for substrates of cytochrome P450-debrisoquine (2D6). Chem. Res. Toxicol. 5:211219, 1992.

52. F. Broly, N. Vandamme, C. Libersa, and M. Lhermitte. The metabolism of mexiletine in relation to the debrisoquine/sparteine-type polymorphism of drug oxidation. Br. J. Clin. Pharmacol. 32:459466, 1991.

53. J.-M. Treluyer, E. Jacqz-Aigrain, F. Alvarez, and T. Cresteil. Expression of CYP2D6 in developing human liver. Eur. J. Biochem. 202:583588, 1991.

54. G. Alván, P. Bechtel, L. Iselius, and U. Gundert-Remy. Hydroxylation polymorphisms of debrisoquine and mephenytoin in European populations. Eur. J. Clin. Pharmacol. 39:533537, 1990.

55. F. Broly, C. Libersa, M. Lhermitte, and B. Dupuis. Inhibitory studies of mexiletine and

dextromethorphan oxidation in human liver microsomes. Biochem. Pharmacol. 39:10451053, 1990.

56. G. B. Faccini, V. Puchetti, and N. Zatti. Dextromethorphan oxidation phenotypes as markers for susceptibility to lung cancer. Clin. Chem. 36:387, 1990.

57. R. J. Guttendorf, M. Britto, R. A. Blouin, T. S. Foster, W. John, K. A. Pittman, and P. J. Wedlund. Rapid screening for polymorphisms in dextromethorphan and mephenytoin metabolism. Br. J. Clin. Pharmacol. 29:373380, 1990.

58. W. E. Haefeli, M. J. Bargetzi, F. Follath, and U. A. Meyer. Potent inhibition of cytochrome P450IID6 (debrisoquin 4-hydroxylase) by flecainide in vitro and in vivo. J. Cardiovasc. Pharmacol. 15:776779, 1990.

59. E. Jacqz-Aigrain, J. Laurent, and F. Alvarez. Dextromethorphan phenotypes in paediatric patients with autoimmune hepatitis. Br. J. Clin. Pharmacol. 30:153154, 1990.

60. Ö. Mortimer, K. Persson, M. G. Ladona, D. Spalding, U. M. Zanger, U. A. Meyer, and A. Rane. Polymorphic formation of morphine from codeine in poor and extensive metabolizers of dextromethorphan: Relationship to the presence of immunoidentified cytochrome P-450IID1. Clin. Pharmacol. Ther. 47:2735, 1990.

61. P. Dayer, T. Leemann, and R. Striberni. Dextromethorphan O-demethylation in liver microsomes as a prototype reaction to monitor cytochrome P-450 db1 activity. Clin. Pharmacol. Ther. 45:3440, 1989.

62. E. Jacqz-Aigrain, Y. Menard, M. Popon, and H. Mathieu. Dextromethorphan phenotypes determined by high-performance liquid chromatography and fluorescence detection. J. Chromatogr. 495:361363, 1989.

63. V. Kerlan, Y. Dreano, J. P. Bercovici, P. H. Beaune, H. H. Floch, and F. Berthou. Nature of cytochromes P450 involved in the 2-/4-hydroxylations of estradiol in human liver microsomes. Biochem. Pharmacol. 44:17451756, 1992.

64. T. Shiraga, H. Matsuda, K. Nagase, K. Iwasaki, K. Noda, H. Yamazaki, T. Shimada, and Y. Funae. Metabolism of FK506, a potent immunosuppressive agent, by cytochrome P450 3A enzymes in rat, dog and human liver microsomes. Biochem. Pharmacol. 47:727735, 1994.

65. S. H. Vincent, B. V. Karanam, S. K. Painter, and S.-H. L. Chiu. In vitro metabolism of FK-506 in rat, rabbit, and human liver microsomes: Identification of a major metabolite and of cytochrome P450 3A as the major enzymes responsible for its metabolism. Arch. Biochem. Biophys. 294:454460, 1992.

66. T. B. Zeugin, K. Brosen, and U. A. Meyer. Determination of imipramine and seven of its metabolites in human liver microsomes by a high-performance liquid chromatographic method. Anal. Biochem. 189:99102, 1990.

67. E. Skjelbo, and K. Brosen. Inhibitors of imipramine metabolism by human liver microsomes. Br. J. Clin. Pharmacol. 34:256261, 1992.

68. K. Chiba, A. Saitoh, E. Koyama, M. Tani, M. Hayashi, and T. Ishizaki. The role of S-mephenytoin 4'-hydroxylase in imipramine metabolism by human liver microsomes: A two-enzyme kinetic analysis of N-demethylation and 2-hydroxylation. Br. J. Clin. Pharmacol. 37:237242, 1994.

69. Y. Masubuchi, S. Umeda, S. Igarashi, S. Fujita, S. Narimatsu, and T. Suzuki. Participation of the CYP2D subfamily in lidocaine 3-hydroxylation and formation of a reactive metabolite covalently bound to liver microsomal protein in rats. Biochem.

Pharmacol. 46:18671869, 1993.

70. S. Imaoka, K. Enomoto, Y. Oda, A. Asada, M. Fujimori, T. Shimada, S. Fujita, F. P. Guengerich, and Y. Funae. Lidocaine metabolism by human cytochrome P-450s purified from hepatic microsomes: Comparison of those with rat hepatic cytochrome P-450s. J. Pharmacol. Exp. Ther. 255:13851391, 1990.

71. M. J. Bargetzi, T. Aoyama, F. J. Gonzalez, and U.A. Meyer. Lidocaine metabolism in human liver microsomes by cytochrome P450IIIA4. Clin Pharmacol. Ther. 46:521527, 1989.

72. R. W. Wang, P. H. Kari, A. Y. H. Lu, P. E. Thomas, F. P. Guengerich, and K. P. Vyas. Biotransformation of lovastatin. IV. Identification of cytochrome P450 3A proteins as the major enzymes responsible for the oxidative metabolism of lovastatin in rat and human liver microsomes. Arch. Biochem. Biophys. 290:355361, 1991.

73. J. A. Goldstein, M. B. Faletto, M. Romkes-Sparks, T. Sullivan, S. Kitareewan, J. L. Raucy, J. M. Lasker, and B. I. Ghanayem. Evidence that CYP2C19 is the major (S)-mephenytoin 4'-hydroxylase in humans. Biochemistry 33:17431752, 1994.

74. T. Andersson, J. O. Miners, M. E. Veronese, W. Tassaneeyakul, U. A. Meyer, and D. J. Birkett. Identification of human liver cytochrome P450 isoforms mediating omeprazole metabolism. Br. J. Clin. Pharmacol. 36:521530, 1993.

75. C. Funck-Brentano, O. Bosco, E. Jacqz-Aigrain, A. Keundjian, and P. Jaillon. Relation between chloroguanide bioactivation to cycloguanil and the genetically determined metabolism of mephenytoin in humans. Clin. Pharmacol. Ther. 51:507512, 1992.

76. P. M. Dansette, C. Amar, P. Valadon, C. Pons, P. H. Beaune, and D. Mansuy. Hydroxylation and formation of electrophilic metabolites of tienilic acid and its isomer by human liver microsomes. Catalysis by a cytochrome P450 IIC different from that responsible for mephenytoin hydroxylation. Biochem. Pharmacol. 41:553560, 1991.

77. M. Romkes, M. B. Faletto, J. A. Blaisdell, J. L. Raucy, and J. A. Goldstein. Cloning and expression of complementary DNAs for multiple members of the human cytochrome P450IIC subfamily. Biochemistry 30:32473255, 1991.

78. J. H. M. Schellens, H. Ghabrial, H. H. F. Van der Wart, E. N. Bakker, G. R. Wilkinson, and D. D. Breimer. Differential effects of quinidine on the disposition of nifedipine, sparteine, and mephenytoin in humans. Clin. Pharmacol. Ther. 50:520528, 1991.

79. P. K. Srivastava, C.-H. Yun, P. H. Beaune, C. Ged, and F. P. Guengerich. Separation of human liver microsomal tolbutamide hydroxylase and (S)-mephenytoin 4'-hydroxylase cytochrome P-450 enzymes. Mol. Pharmacol. 40:6979, 1991.

80. T. Yasumori, Y. Yamazoe, and R. Kato. Cytochrome P-450 human-2 (P-450IIC9) in mephenytoin hydroxylation polymorphism in human livers: Differences in substrate and stereoselectivities among microheterogeneous P-450IIC species expressed in yeasts. J. Biochem. (Tokyo) 109:711717, 1991.

81. N. A. Helsby, S. A. Ward, R. E. Howells, and A. M. Breckenridge. In vitro metabolism of the biguanide antimalarials in human liver microsomes: Evidence for a role of the mephenytoin hydroxylase (P450 MP) enzyme. Br. J. Clin. Pharmacol. 30:287291, 1990.

82. M. V. Relling, T. Aoyama, F. J. Gonzalez, and U. A. Meyer. Tolbutamide and mephenytoin hydroxylation by human cytochrome P450s in the CYP2C subfamily. J. Pharmacol. Exp. Ther. 252:442447, 1990.

83. T. Yasumori, N. Murayama, Y. Yamazoe, and R. Kato. Polymorphism in

hydroxylation of mephenytoin and hexobarbital stereoisomers in relation to hepatic P-450 human-2. Clin. Pharmacol. Ther. 47:313322, 1990.

84. W. R. Brian, C. Ged, T. M. Bellew, P. K. Srivastava, R. W. Bork, D. R. Umbenhauer, R. S. Lloyd, and F. P. Guengerich. Human liver mephenytoin 4'-hydroxylase cytochrome P-450 proteins and genes. Drug Metab. Rev. 20:449465, 1989.

85. T. Yasumori, N. Murayama, Y. Yamazoe, A. Abe, Y. Nogi, T. Fukasawa, and R. Kato. Expression of a human P-450IIC gene in yeast cells using galactose-inducible expression system. Mol. Pharmacol. 35:443449, 1989.

86. R. G. Knodell, R. K. Dubey, G. R. Wilkinson, and F. P. Guengerich. Oxidative metabolism of hexobarbital in human liver: Relationship to polymorphic S-mephenytoin 4-hydroxylation. J. Pharmacol. Exp. Ther. 245:845849, 1988.

87. R. R. Meehan, J. R. Gosden, D. Rout, N. D. Hastie, T. Friedberg, M. Adesnik, R. Buckland, V. van Heyningen, J. Fletcher, N. D. Spurr, J. Sweeney, and C. R. Wolf. Human cytochrome P-450 PB-1: A multigene family involved in mephenytoin and steroid oxidations that maps to chromosome 10. Am. J. Hum. Genet. 42:2637, 1988.

88. S. D. Hall, F. P. Guengerich, R. A. Branch, and G. R. Wilkinson. Characterization and inhibition of mephenytoin 4-hydroxylase activity in human liver microsomes. J. Pharmacol. Exp. Ther. 240:216222, 1987.

89. T. Shimada, K. S. Misono, and F. P. Guengerich. Human liver microsomal cytochrome P-450 mephenytoin 4-hydroxylase, a prototype of genetic polymorphism in oxidative drug metabolism. Purification and characterization of two similar forms involved in the reaction. J. Biol. Chem. 261:909921, 1986.

90. T. Inaba, M. Jurima, W. A. Mahon, and W. Kalow. In vitro inhibition studies of two isozymes of human liver cytochrome P-450. Mephenytoin p-hydroxylase and sparteine monooxygenase. Drug Metab. Dispos. 13:443448, 1985.

91. U. T. Meier, T. Kronbach, and U. A. Meyer. Assay of mephenytoin metabolism in human liver microsomes by high-performance liquid chromatography. Anal. Biochem. 151:286291, 1985.

92. T. Shimada, J. P. Shea, and F. P. Guengerich. A convenient assay for mephenytoin 4-hydroxylase activity of human liver microsomal cytochrome P-450. Anal. Biochem. 147:174179, 1985.

93. A. Kupfer, R. Patwardhan, S. Ward, S. Schenker, R. Preisig, and R. A. Branch. Stereoselective metabolism and pharmacogenetic control of 5-phenyl-5-ethylhy-dantion (nirvanol) in humans. J. Pharmacol. Exp. Ther. 230:2833, 1984.

94. A. Kupfer, P. V. Desmond, S. Schenker, and R. A. Branch. Stereoselective metabolism and disposition of the enantiomers of mephenytoin during chronic oral administration of the racemic drug in man. J. Pharmacol. Exp. Ther. 221:590597, 1982.

95. N. Vandamme, F. Broly, C. Libersa, C. Courseau, and M. Lhermitte. Stereoselective hydroxylation of mexiletine in human liver microsomes: Implication of P450IID6a preliminary report. J. Cardiovasc. Pharmacol. 21:7783, 1993.

96. M.-P. Gascon, and P. Dayer. In vitro forecasting of drugs which may interfere with the

biotransformation of midazolam. Eur. J. Clin. Pharmacol. 41:573578, 1991.

97. G. Li, G. Treiber, J. Meinshausen, J. Wolf, J. Werringloer, and U. Klotz. Is cyclosporin A an inhibitor of drug metabolism? Br. J. Clin. Pharmacol. 30:7177, 1990.

98. T. Kronbach, D. Mathys, M. Umeno, F. J. Gonzalez, and U. A. Meyer. Oxidation of midazolam and triazolam by human liver cytochrome P450IIIA4. Mol. Pharmacol. 36:8996, 1989.

99. R. W. Bork, T. Muto, P. H. Beaune, P. K. Srivastava, R. S. Lloyd, and F. P. Guengerich. Characterization of mRNA species related to human liver cytochrome P-450 nifedipine oxidase and the regulation of catalytic activity. J. Biol. Chem. 264:910919, 1989.

100. T. Shimada, and F. P. Guengerich. Evidence for cytochrome P-450NF, the nifedipine oxidase, being the principal enzyme involved in the bioactivation of aflatoxins in human liver. Proc. Natl. Acad. Sci. USA 86:462465, 1989.

101. F. J. Gonzalez, B. J. Schmid, M. Umeno, O. W. McBride, J. P. Hardwick, U. A. Meyer, H. V. Gelboin, and J. R. Idle. Human P450PCN1: Sequence, chromosome localization, and direct evidence through cDNA expression that P450PCN1 is nifedipine oxidase. DNA 7:7986, 1988.

102. M. Komori, T. Hashizume, H. Ohi, T. Miura, M. Kitada, K. Nagashima, and T. Kamataki. Cytochrome P-450 in human liver microsomes: High-performance liquid chromatographic isolation of three forms and their characterization. J. Biochem. (Tokyo) 104:912916, 1988.

103. P. H. Beaune, D. R. Umbenhauer, R. W. Bork, R. S. Lloyd, and F. P. Guengerich. Isolation and sequence determination of a cDNA clone related to human cytochrome P-450 nifedipine oxidase. Proc. Natl. Acad. Sci. USA 83:80648068, 1986.

104. F. P. Guengerich, M. V. Martin, P. H. Beaune, P. Kremers, T. Wolff, and D. J. Waxman. Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. J. Biol. Chem. 261:50515060, 1986.

105. B. W. LeDuc, P. R. Sinclair, L. Shuster, J. F. Sinclair, J. E. Evans, and D. J. Greenblatt. Norcocaine and N-hydroxynorcocaine formation in human liver microsomes: role of cytochrome P4503A4. Pharmacology 46:294300, 1993.

106. K. Chiba, K. Kobayashi, K. Manabe, M. Tani, T. Kamataki, and T. Ishizaki. Oxidative metabolism of omeprazole in human liver microsomes: Cosegregation with S-mephenytoin 4'-hydroxylation. J. Pharmacol. Exp. Ther. 266:5259, 1993.

107. C. J. Doecke, L. N. Sansom, and M. E. McManus. Phenytoin 4-hydroxylation by rabbit liver P450IIC3 and identification of orthologs in human liver microsomes. Biochem. Biophys. Res. Commun. 166:860866, 1990.

108. C. J. Doecke, M. E. Veronese, S. M. Pond, J. O. Miners, D. J. Birkett, L. N. Sansom, and M. E. McManus. Relationship between phenytoin and tolbutamide hydroxylations in human liver microsomes. Br. J. Clin. Pharmacol. 31:125130, 1991.

109. U. Fuhr, E.-M. Anders, G. Mahr, F. Sörgel, and A. H. Staib. Inhibitory potency of quinolone antibacterial agents against cytochrome P450IA2 activity in vivo and in vitro. Antimicrob. Agents Chemother. 36:942948, 1992.

110. S. V. Otton, R. F. Tyndale, D. Wu, T. Inaba, W. Kalow, and E. M. Sellers. Catalytic and immunologic similarities between monkey and human liver cytochrome P-450db1 (human cytochrome P-450 2D6). Drug Metab. Dispos. 20:15, 1992.

111. T. F. Woolf, W. F. Pool, S. M. Bjorge, T. Chang, O. P. Goel, C. F. Purchase, II, M. C. Schroeder, K. L. Kunze, and W. F. Trager. Bioactivation and irreversible binding of the cognition activator tacrine using human and rat liver microsomal preparations: Species difference. Drug Metab. Dispos. 21:874882, 1993.

112. E. A. Blankson, S. W. Ellis, M. S. Lennard, G. T. Tucker, and K. Rogers. The metabolism of tamoxifen by human liver microsomes is not mediated by cytochrome P450IID6. Biochem. Pharmacol. 42 Suppl.:S209S212, 1991.

113. T. Cresteil, B. Monsarrat, P. Alvinerie, J.M. Tréluyer, I. Vieira, and M. Wright. Taxol metabolism by human liver microsomes: Identification of cytochrome P450 isozymes involved in its biotransformation. Cancer Res. 54:386392, 1994.

114. M. A. Sarkar, C. Hunt, P. S. Guzelian, and H. T. Karnes. Characterization of human liver cytochromes P-450 involved in theophylline metabolism. Drug Metab. Dispos. 20:3137, 1992.

115. P. Beaune, P. M. Dansette, D. Mansuy, L. Kiffel, M. Finck, C. Amar, J. P. Leroux, and J. C. Homberg. Human anti-endoplasmic reticulum autoantibodies appearing in a drug-induced hepatitis are directed against a human liver cytochrome P-450 that hydroxlates the drug. Proc. Natl. Acad. Sci. USA 84:551555, 1987.

116. C. Pons, P. M. Dansette, C. Amar, M. Jaouen, C. R. Wolf, J. Gregeois, J. C. Homberg, and D. Mansuy. Detection of human hepatitis anti-liver kidney microsomes (LKM2) autoantibodies on rat liver sections is predominantly due to reactivity with rat liver P-450 IIC11. J. Pharmacol. Exp. Ther. 259:13281334, 1991.

117. J. O. Miners, K. J. Smith, R. A. Robson, M. E. McManus, M. Veronese, and D. J. Birkett. Tolbutamide hydroxylation by human liver microsomes. Biochem. Pharmacol. 37:11371144, 1988.

118. M. E. Veronese, M. E. McManus, P. Laupattarakasem, J. O. Miners, and D. J. Birkett. Tolbutamide hydroxylation by human, rabbit and rat liver microsomes and by purified forms of cytochrome P-450. Drug Metab. Dispos. 18:356361, 1990.

119. H. K. Kroemer, H. Echizen, H. Heidemann, and M. Eichelbaum. Predictability of the in vivo metabolism of verapamil from in vitro data: Contribution of individual metabolic pathways and stereoselective aspects. J. Pharmacol. Exp. Ther. 260:10521057, 1992.

120. P. P. Wang, P. Beaune, L. S. Kaminsky, G. A. Dannan, F. F. Kadlubar, D. Larrey, and F. P. Guengerich. Purification and characterization of six cytochrome P-450 isozymes from human liver microsomes. Biochemistry 22:53755383, 1983.

121. L. S. Kaminsky, D. A. Dunbar, P. P. Wang, P. Beaune, D. Larrey, F. P. Guengerich, R. G. Schnellmann, and I. G. Sipes. Human hepatic cytochrome P-450 composition as probed by in vitro microsomal metabolism of warfarin. Drug Metab. Dispos. 12:470477, 1984.

122. P. H. Beaune, P. G. Kremers, L. S. Kaminsky, J. DeGraeve, A. Albert, and F. P. Guengerich. Comparison of monooxygenase activities and cytochrome P-450 isozyme concentrations in human liver microsomes. Drug Metab. Dispos. 14:437442, 1986.

123. D. R. Abernethy, L. S. Kaminsky, and T. H. Dickinson. Selective inhibition of warfarin metabolism by diltiazem in humans. J. Pharmacol. Exp. Ther. 257:411415, 1991.

124. H. Nakasa, M. Komiya, S. Ohmori, T. Rikihisa, M. Kiuchi, and M. Kitada. Characterization of human liver microsomal cytochrome P450 involved in the reductive metabolism of zonisamide. Mol. Pharmacol. 44:216221, 1993.

125. Y. Gluzman. SV40-transformed simian cells support the replication of early SV40 mutants. Cell 23:175182, 1981.

126. L. M. Sompayrac, and K. J. Danna. Efficient infection of monkey cells with DNA of simian virus 40. Proc. Natl. Acad. Sci. USA 78:75757578, 1981.

127. M. X. Zuber, E. R. Simpson, and M. R. Waterman. Expression of bovine 17a-hydroxylase cytochrome P-450 cDNA in nonsteroidogenic (COS1) cells. Science 234:12581261, 1986.

128. T. H. Manoharan, R. B. Puchalski, J. A. Burgess, C. B. Pickett, and W. E. Fahl. Promoter-glutathione S-transferase Ya cDNA hybrid genes. Expression and conferred resistance to an alkylating molecule in mammalian cells. J. Biol. Chem. 262:37393745, 1987.

129. C. J. Corbin, S. Graham-Lorence, M. McPhaul, J. I. Mason, C. R. Mendelson, and E. R. Simpson. Isolation of a full-length cDNA insert encoding human aromatase system cytochrome P-450 and its expression in nonsteroidogenic cells. Proc. Natl. Acad. Sci. USA 85:89488952, 1988.

130. D. Harding, S. Fournel-Gigleux, M. R. Jackson, and B. Burchell. Cloning and substrate specificity of a human phenol UDP-glucuronosyltransferase expressed in COS-7 cells. Proc. Natl. Acad. Sci. USA 85:83818385, 1988.

131. M. R. Jackson, S. Fournel-Gigleux, D. Harding, and B. Burchell. Examination of the substrate specificity of cloned rat kidney phenol UDP-glucoronyltransferase expresses in COS-7 cells. Mol. Pharmacol. 34:638642, 1988.

132. M. X. Zuber, J. I. Mason, E. R. Simpson, and M. R. Waterman. Simultaneous transfection of COS-1 cells with mitochondrial and microsomal steroid hydroxylases: Incorporation of a steroidogenic pathway into nonsteroidogenic cells. Proc. Natl. Acad. Sci. USA 85:699703, 1988.

133. P. Swart, R. W. Estabrook, J. I. Mason, and M. R. Waterman. Catalytic activity of human and bovine adrenal cytochromes P-45017$_{a,lyase}$ expressed in Cos 1 cells. Biochem. Soc. Trans. 17:10251026, 1989.

134. O. Minowa, K. Sogawa, Y. Higashi, and Y. Fujii-Kuriyama. Functional expression of microsomal and mitochondrial cytochrome P-450 (d and SCC) in COS-7 cells from cloned cDNA. Cell Struct. Funct. 15:2130, 1990.

135. K. Morohashi, Y. Nonaka, S. Kirita, O. Hatano, A. Takakusu, M. Okamoto, and T. Omura. Enzymatic activities of P-450(11b)s expressed by two cDNAs in COS-7 cells. J. Biochem. (Tokyo) 107:635640, 1990.

136. J. M. Trant, M. C. Lorence, E. F. Johnson, C. H. L. Shackleton, J. I. Mason, and R. W. Estabrook. Characterization of the steroid-metabolizing capacity of the hepatic cytochrome P450IIC5 expressed in COS-1 cells: 3b-Hydroxysteroid

dehydrogenase/delta-4 isomerase type activity. Proc. Natl. Acad. Sci. USA 87:97569760, 1990.

137. B. J. Clark, and M. R. Waterman. Heterologous expression of mammalian P450 in COS cells. Methods Enzymol. 206:100108, 1991.

138. K. M. Curnow, M.-T. Tusie-Luna, L. Pascoe, R. Natarajan, J.-L. Gu, J. L. Nadler, and P. C. White. The product of the CYP11B2 gene is required for aldosterone biosynthesis in the human adrenal cortex. Mol. Endocrinol. 5:15131522, 1991.

139. Y. Higashi, and Y. Fujii-Kuriyama. Functional analysis of mutant P450(C21) genes in COS cell expression system. Methods Enzymol. 206:166173, 1991.

140. M. P. Lawton, T. Kronbach, E. F. Johnson, and R. M. Philpot. Properties of expressed and native flavin-containing monooxygenases: Evidence of multiple forms in rabbit liver and lung. Mol. Pharmacol. 40:692698, 1991.

141. Y. Nonaka, and M. Okamoto. Functional expression of the cDNAs encoding rat 11b-hydroxylase [cytochrome P450(11b)] and aldosterone synthase [cytochrome P450(11b, aldo)]. Eur. J. Biochem. 202:897902, 1991.

142. M. E. Veronese, P. I. Mackenzie, C. J. Doecke, M. E. McManus, J. O. Miners, and D. J. Birkett. Tolbutamide and phenytoin hydroxylations by cDNA-expressed human liver cytochrome P4502C9. Biochem. Biophys. Res. Commun. 175:11121118, 1991.

143. N. Harada, H. Ogawa, M. Shozu, K. Yamada, K. Suhara, E. Nishida, and Y. Takagi. Biochemical and molecular genetic analyses on placental aromatase (P-450AROM) deficiency. J. Biol. Chem. 267:47814785, 1992.

144. T. Kawamoto, Y. Mitsuuchi, K. Toda, Y. Yokoyama, K. Miyahara, S. Miura, T. Ohnishi, Y. Ichikawa, K. Nakao, H. Imura, S. Ulick, and Y. Shizuta. Role of steroid 11b-hydroxylase and steroid 18-hydroxylase in the biosynthesis of glucocorticoids and mineralocorticoids in humans. Proc. Natl. Acad. Sci. USA 89:14581462, 1992.

145. Y. Nonaka, M. Okamoto, K. Morohashi, S. Kirita, T. Hashimoto, and T. Omura. Functional expression of cDNAs for bovine 11b-hydroxylase-aldosterone synthases, P450(11b)-2 and -3 and their chimeras. J. Steroid Biochem. Mol. Biol. 41:779780, 1992.

146. S. Monno, H. Ogawa, T. Date, M. Fujioka, W. L. Miller, and M. Kobayashi. Mutation of histidine 373 to leucine in cytochrome P450c17 causes 17a-hydroxylase deficiency. J. Biol. Chem. 268:2581125817, 1993.

147. M. E. McManus, W. M. Burgess, M. E. Veronese, A. Huggett, L. C. Quattrochi, and R. H. Tukey. Metabolism of 2-acetylaminofluorene, benzo(a)pyrene and activation of food derived heterocyclic amine mutagens by human cytochromes P-450. Cancer Res. 50:33673376, 1990.

148. E. F. Johnson, T. Kronbach, and M.-H. Hsu. Analysis of the catalytic specificity of cytochrome P450 enzymes through site directed mutagenesis. FASEB J 6:700705, 1992.

149. T. Kronbach, T. M. Larabee, and E. F. Johnson. Hybrid cytochromes P-450 identify a substrate binding domain in P-450IIC5 and P-450IIC4. Proc. Natl. Acad. Sci. USA 86:82628265, 1989.

150. M. Noshiro and K. Okuda. Molecular cloning and sequence analysis of cDNA encoding human cholesterol 7a-hydroxylase. FEBS Lett. 268:137140, 1990.

151. N. Matsukawa, Y. Nonaka, Z. Ying, J. Higaki, T. Ogihara, and M. Okamoto. Molecular cloning and expression of cDNAs encoding rat aldosterone synthase: Variants of cytochrome P-45011b. Biochem. Biophys. Res. Commun. 169:245252, 1990.

152. L. Brodin and O. Shupliakov. Functional diversity of central glutamate synapsespre- and post-synaptic mechanisms. Acta Physiol. Scand. 150:110, 1994.

153. M. C. Lorence, J. M. Trant, J. I. Mason, C. R. Bhasker, Y. Fujii-Kuriyama, R. W. Estabrook, and M. W. Waterman. Expression of a full-length cDNA encoding bovine adrenal cytochrome P450c21. Arch. Biochem. Biophys. 273:7988, 1989.

154. S. Imaoka, H. Ogawa, S. Kimura, and F. J. Gonzalez. Complete cDNA sequence and cDNA-directed expression of CYP4A11, a fatty acid omega-hydroxylase expressed in human kidney. DNA Cell Biol. 12:893899, 1993.

155. T. Aoyama, S. Yamano, D. J. Waxman, D. P. Lapenson, U. A. Meyer, V. Fischer, R. Tyndale, T. Inaba, W. Kalow, H. V. Gelboin, and F. J. Gonzalez. Cytochrome P-450 hPCN3, a novel cytochrome P-450 IIIA gene product that is differentially expressed in adult human liver. J. Biol. Chem. 264:1038810395, 1989.

156. T. Aoyama, K. Korzekwa, K. Nagata, J. Gillette, H. V. Gelboin, and F. J. Gonzales. cDNA-directed expression of rat testosterone 7a-hydroxylase using the modified vaccinia virus, T7-RNA-polymerase system and evidence for 6a-hydroxylation and delta6-testosterone formation. Eur. J. Biochem. 181:331336, 1989.

157. N. Battula. Transduction of cytochrome P3-450 by retroviruses: Constitutive expression of enzymatically active microsomal hemoprotein in animal cells. J. Biol. Chem. 264:29912996, 1989.

158. T. Aoyama, S. Yamano, P. S. Guzelian, H. V. Gelboin, and F. J. Gonzalez. Five of 12 forms of vaccinia virus-expressed human hepatic cytochrome P450 metabolically activate aflatoxin B_1. Proc. Natl. Acad. Sci. USA 87:47904793, 1990.

159. T. Aoyama, K. Nagata, Y. Yamazoe, R. Kato, E. Matsunaga, H. V. Gelboin, and F. J. Gonzalez. Cytochrome b_5 potentiation of cytochrome P-450 catalytic activity demonstrated by a vaccinia virus-mediated in situ reconstitution system. Proc. Natl. Acad. Sci. USA 87:54255429, 1990.

160. T. Aoyama, K. Korzekwa, T. Matsunaga, K. Nagata, J. Gillette, H. V. Gelboin, and F. J. Gonzalez. cDNA-directed expression of rat P450s IIA1 and IIA2: Catalytic activities toward steroids and xenobiotics and comparison with the enzymes purified from liver. Drug Metab. Dispos. 18:378382, 1990.

161. E. Matsunaga, T. Zeugin, U. M. Zanger, T. Aoyma, U. A. Meyer, and F. J. Gonzalez. Sequence requirements for cytochrome P-450IID1 catalytic activity: A single amino acid change (ILE380PHE) specifically decreases Vmax of the enzyme for bufuralol but not debrisoquine hydroxylation. J. Biol. Chem. 265:1719717201, 1990.

162. F. J. Gonzalez, T. Aoyama, and H. V. Gelboin. Expression of mammalian cytochrome P450 using vaccinia virus. Methods Enzymol. 206:8592, 1991.

163. N. Hanioka, F. J. Gonzalez, N. A. Lindberg, G. Liu, H. V. Gelboin, and K. R. Korzekwa. Site-directed mutagenesis of cytochrome P450s CYP2A1 and CYP2A2: Influence of the distal helix on the kinetics of testosterone hydroxylation. Biochemistry 31:33643370, 1992.

164. I. Goldfarb, K. Korzekwa, K. W. Krausz, F. Gonzalez, and H. V. Gelboin. Cross-

reactivity of thirteen monoclonal antibodies with ten vaccinia cDNA expressed rat, mouse and human cytochrome P450s. Biochem. Pharmacol. 46:787790, 1993.

165. F. J. Gonzalez. Molecular biology of human xenobiotic-metabolizing cytochromes P450: Role of vaccinia virus cDNA expression in evaluating catalytic function. Toxicology 82:7788, 1993.

166. C. L. Crespi and W. G. Thilly. Assay for gene mutation in a human lymphoblast line, AHH-1, competent for xenobiotic metabolism. Mutat. Res. 128:221230, 1984.

167. C. L. Crespi, J. D. Altman, and M. A. Marletta. Xenobiotic metabolism and mutation in a human lymphoblastoid cell line. Chem. Biol. Interact. 53:257272, 1985.

168. F. K. Friedman, R. C. Robinson, B. J. Song, S. S. Park, C. L. Crespi, M. A. Marletta, and H. V. Gelboin. Monoclonal antibody-directed determination of cytochrome P-450 types expressed in a human lymphoblastoid cell line. Mol. Pharmacol. 27:652655, 1985.

169. C. L. Crespi, R. Langenbach, K. Rudo, Y. T. Chem, and R. L. Davies. Transfection of a human cytochrome P-450 gene into the human lymphoblastoid cell line, AHH-1, and use of the recombinant cell line in gene mutation assays. Carcinogenesis 10:295301, 1989.

170. C. L. Crespi, D. T. Steimel, T. Aoyama, H. V. Gelboin, and F. J. Gonzalez. Stable expression of human cytochrome P450IA2 cDNA in a human lymphoblastoid cell line: Role of the enzyme in the metabolic activation of aflatoxin B1. Mol. Carcinog. 3:58, 1990.

171. C. L. Crespi, B. W. Penman, J. A. E. Leakey, M. P. Arlotto, A. Stark, A. Parkinson, T. Turner, D. T. Steimel, K. Rudo, R. L. Davies, and R. Langenbach. 1990. Human cytochrome P450IIA3: cDNA sequence, role of the enzyme in the metabolic activation of promutagens, comparison to nitrosamine activation by human cytochrome P450IIE1. Carcinogenesis 11:12931300, 1990.

172. C. L. Crespi, B. W. Penman, D. T. Steimel, H. V. Gelboin, and F. J. Gonzalez. The development of a human cell line stably expressing human CYP3A4: Role in the metabolic activation of aflatoxin B1 and comparison to CYP1A2 and CYP2A3. Carcinogenesis 12:355359, 1991.

173. C. L. Crespi. Expression of cytochrome P450 cDNAs in human B lymphoblastoid cells: Applications to toxicology and metabolite analysis. Methods Enzymol. 206:123129, 1991.

174. C. L. Crespi, B. W. Penman, H. V. Gelboin, and F. J. Gonzalez. A tobacco smoke-derived nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, is activated by multiple human cytochrome P450s including the polymorphic human cytochrome P4502D6. Carcinogenesis 12:11971201, 1991.

175. F. J. Gonzalez, C. L. Crespi, and H. V. Gelboin. cDNA-expressed human cytochrome P450s: A new age of molecular toxicology and human risk assessment. Mutat. Res. 247:113127, 1991.

176. C. L. Crespi, R. Langenbach, and B. W. Penman. Human cell lines, derived from AHH-1 TK+/-human lymphoblasts, genetically engineered for expression of cytochromes P450. Toxicology 82:89104, 1993.

177. C. L. Crespi, B. W. Penman, F. J. Gonzalez, H. V. Gelboin, M. Galvin, and R. Langenbach. Genetic toxicology using human cell lines expressing human P-450.

Biochem. Soc. Trans. 21:10231028, 1993.

178. C. L. Crespi, F. J. Gonzalez, D. T. Steimel, T. R. Turner, H. V. Gelboin, B. W. Penman, and R. Langenbach. A metabolically competent human cell line expressing five cDNAs encoding procarcinogen-activating enzymes: Application to mutagenicity testing. Chem. Res. Toxicol. 4:566572, 1993.

179. J. Doehmer, S. Dogra, T. Friedberg, S. Monier, M. Adesnik, H. Glatt, and F. Oesch. Stable expression of rat cytochrome P-450IIB1 cDNA in Chinese hamster cells (V79) and metabolic activation of aflatoxin B1. Proc. Natl. Acad. Sci. USA 85:57695773, 1988.

180. F. Kiefer and F. J. Wiebel. V79 chinese hamster cells express cytochrome P-450 activity after simultaneous exposure to polycyclic aromatic hydrocarbons and aminophylline. Toxicol. Lett. 48:265273, 1989.

181. S. Dogra, J. Doehmer, H. Glatt, H. Molders, P. Siegert, T. Friedberg, A. Seidel, and F. Oesch. Stable expression of rat cytochrome P-450IA1 cDNA in V79 chinese hamster cells and their use in mutagenicity testing. Mol. Pharmacol. 37:608613, 1990.

182. J. Doehmer, and F. Oesch. V79 Chinese hamster cells genetically engineered for stable expression of cytochromes P450. Methods Enzymol. 206:117123, 1991.

183. C. Wölfel, K.-L. Platt, S. Dogra, H. Glatt, F. Wächter, and J. Doehmer. Stable expression of rat cytochrome P450IA2 cDNA and hydroxylation of 17b-estradiol and 2-aminofluorene in V79 Chinese hamster cells. Mol. Carcinog. 4:489498, 1991.

184. U. Fuhr, J. Doehmer, N. Battula, C. Wölfel, C. Kudla, Y. Keita, and A. H. Staib. Biotransformation of caffeine and theophylline in mammalian cell lines genetically engineered for expression of single cytochrome P450 isoforms. Biochem. Pharmacol. 43:225235, 1992.

185. J. Doehmer. V79 Chinese hamster cells genetically engineered for cytochrome P450 and their use in mutagenicity and metabolism studies. Toxicology 82:105118, 1993.

186. U. Fuhr, J. Doehmer, N. Battula, C. Wölfel, I. Flick, C. Kudla, Y. Keita, and A. Horst Staib. Biotransformation of methylxanthines in mammalian cell lines genetically engineered for expression of single cytochrome P450 isoforms. Allocation of metabolic pathways to isoforms and inhibitory effects of quinolones. Toxicology 82:169189, 1993.

187. K. G. Jensen, S. Loft, J. Doehmer, and H. E. Poulsen. Metabolism of phenacetin in V79 Chinese hamster cell cultures expressing rat liver cytochrome P4501A2 compared to isolated rat hepatocytes. Biochem. Pharmacol. 45:11711173, 1993.

188. W. A. Schmalix, H. Mäser, F. Kiefer, R. Reen, F. J. Wiebel, F. Gonzalez, A. Seidel, H. Glatt, H. Greim, and J. Doehmer. Stable expression of human cytochrome P450 1A1 cDNA in V79 Chinese hamster cells and metabolic activation of benzo[a]pyrene. Eur. J. Pharmacol. Environ. Toxicol. Pharmacol. 248:251261, 1993.

189. A. Asseffa, S. J. Smith, K. Nagata, J. Gillette, H. V. Gelboin, and F. J. Gonzalez. Novel exogenous heme-dependent expression of mammalian cytochrome P450 using baculovirus. Arch Biochem. Biophys. 274:481490, 1989.

190. D. Ohta, Y. Matsu-ura, and R. Sato. Expression and characterization of a rabbit liver cytochrome P450 belonging to P450IIB subfamily with the aid of the baculovirus

expression vector system. Biochem. Biophys. Res. Commun. 175:394399, 1991.

191. F. J. Gonzalez, S. Kimura, S. Tamura, and H. V. Gelboin. Expression of mammalian cytochrome P450 using baculovirus. Methods Enzymol. 206:9399, 1991.

192. Y. Takagi, H. Ogawa, N. Harada, H. Shimada, and Y. Ishimura. Expression and transport into mitochrondria of bovine cytochrome P-450(SCC) in insect cells using the baculovirus expression system. Biochem. Biophys. Res. Commun. 184:471477, 1992.

193. S. Imaoka, P. J. Wedlund, H. Ogawa, S. Kimura, F. J. Gonzalez, and H.-Y. Kim. Identification of CYP2C23 expressed in rat kidney as an arachidonic acid epoxygenase. J. Pharmacol. Exp. Ther. 267:10121016, 1993.

194. M. Lähde, H. Raunio, O. Pelkonen, M. Karp, and C. Oker-Blom. Expression of human placental cytochrome P450 aromatase (CYP19) cDNA in insect cells using a luciferase based baculovirus vector. Biochem. Biophys. Res. Commun. 197:15111517, 1993.

195. F. P. Guengerich, W. R. Brian, M.-A. Sari, and J. T. Ross. Expression of mammalian cytochrome P450 enzymes using yeast-based vectors. Methods Enzymol. 206:130145, 1991.

196. F. P. Guengerich, E. M. J. Gillam, S. Ohmori, P. Sandhu, W. R. Brian, M.-A. Sari, and M. Iwasaki. Expression of human cytochrome P450 enzymes in yeast and bacteria and relevance to studies on catalytic specificity. Toxicology 82:2137, 1993.

197. P. Beaune, M. Bourdi, C. Belloc, J.-C. Gautier, F. P. Guengerich, and P. Valadon. Immunotoxicology and expression of human cytochrome P450 in microorganisms. Toxicology 82:5360, 1993.

198. J. P. Renaud, M. A. Peyronneau, P. Urban, G. Truan, C. Cullin, D. Pompon, P. Beaune, and D. Mansuy. Recombinant yeast in drug metabolism. Toxicology 82:3952, 1993.

199. P. Urban, G. Truan, J.-C. Gautier, and D. Pompon. Xenobiotic metabolism in humanized yeast: Engineered yeast cells producing human NADPH-cytochrome P-450 reductase, cytochrome b5, epoxide hydrolase and P-450s. Biochem. Soc. Trans. 21:10281034, 1993.

200. A. Wiseman. Genetically-engineered mammalian cytochromes P-450 from yeastspotential applications. TIBTECH 11:131136, 1993.

201. D. M. Davies, E. F. Johnson, H. H. Liem, and U. Muller-Eberhard. The role of cytosolic proteins in the intracellular transport of haem in rat liver. Biochem. J. 202:211216, 1982.

202. T. Sakaki, K. Oeda, M. Miyoshi, and H. Ohkawa. Characterization of rat cytochrome P-450mc synthesized in saccharomyces cerevisiae. J. Biochem. 98:167175, 1985.

203. T. Sakaki, K. Oeda, Y. Yabusaki, and H. Ohkawa. Monooxygenase activity of Saccharomyces cerevisiae cells transformed with expression plasmids carrying rat cytochrome P-450 cDNA. J. Biochem. 99:741749, 1986.

204. T. Shimizu, K. Sogawa, Y. Fujii-Kuriyama, M. Takahashi, Y. Ogoma, and M. Hatano. Expression of cytochrome P-450 d by Saccharomyces cerevisiae. FEBS Lett. 207:217221, 1986.

205. H. Murakami, Y. Yabusaki, T. Sakaki, M. Shibata, and H. Ohkawa. A genetically engineered P450 monooxygenase: Construction of the functional fused enzyme between rat cytochrome P450c and NADPH-cytochrome P450 reductase. DNA 6:189197, 1987.

206. Y. Imai. Cytochrome P-450 related to P-450 4 from phenobarbital-treated rabbit liver: Molecular cloning of cDNA and Characterization of cytochrome P-450 obtained by its expression in yeast cells. J. Biochem. 101:11291139, 1987.

207. T. Sakaki, M. Shibata, Y. Yabusaki, and H. Ohkawa. Expression in Saccharomyces cerevisiae of chimeric cytochrome P450 cDNAs constructed from cDNAs for rat cytochrome P450c and P450d. DNA 6:3139, 1987.

208. C. Cullin, and D. Pompon. Synthesis of functional mouse cytochromes P-450 P1 and chimeric P-450 P3-1 in the yeast Saccharomyces cerevisiae. Gene 65:203217, 1988.

209. S. I. Hayashi, K. Morohashi, H. Yoshioka, K. Okuda, and T. Omura. Expression of a rat liver microsomal cytochrome P-450 catalyzing testosterone 16a-hydroxylation in Saccharomyces cerevisiae: Vitamin D3 25-hydroxylase and testosterone 16a-hydroxylase are distinct forms of cytochrome P-450. J. Biochem. 103:858862, 1988.

210. D. Pompon. cDNA cloning and functional expression in yeast Saccharomyces cerevisiae of b-naphthoflavone-induced rabbit liver P-450 LM4 and LM6. Eur. J. Biochem. 177:285293, 1988.

211. T. Shimizu, K. Hirano, M. Takahashi, M. Hatano, and Y. Fujii-Kuriyama. Site-directed mutageneses of rat liver cytochrome P-450d: Axial ligand and heme incorporation. Biochemistry 27:41384141, 1988.

212. Y. Yabusaki, H. Murakami, T. Sakaki, M. Shibata, and H. Ohkawa. Genetically engineered modification of P450 monooxygenases: Functional analysis of the amino-terminal hydrophobic region and hinge region of the P450/reductase fused enzyme. DNA 7:701711, 1988.

213. T. Iwase, T. Sakaki, Y. Yabusaki, H. Ohkawa, Y. Ohta, and S. Kawato. Rotation and interactions of genetically expressed cytochrome P-450IA1 and NADPH-cytochrome P-450 reductase in yeast microsomes. Biochemistry 30:83478351, 1991.

214. T. Shimizu, A. J. M. Sadeque, G. N. Sadeque, M. Hatano, and Y. Fujii-Kuriyama. Ligand binding studies of engineered cytochrome P-450d wild type, proximal mutants, and distal mutants. Biochemistry 30:14901496, 1991.

215. M. Komori, O. Kikuchi, M. Kitada, and T. Kamataki. Molecular cloning of monkey P450 1A1 cDNA and expression in yeast. Biochim. Biophys. Acta 1131:2329, 1992.

216. A. Berson, C. Wolf, C. Chachaty, C. Fisch, D. Fau, D. Eugene, J. Loeper, J.-C. Gauthier, P. Beaune, D. Pompon, P. Maurel, and D. Pessayre. Metabolic activation of the nitroaromatic antiandrogen Flutamide by rat and human cytochromes P-450, including forms belonging to the 3A and 1A subfamilies. J. Pharmacol. Exp. Ther. 265:366372, 1993.

217. V. Carriere, T. Goasduff, D. Ratanasavanh, F. Morel, J.-C. Gautier, A. Guillouzo, P.

Beaune, and F. Berthou. Both cytochromes P450 2E1 and 1A1 are involved in the metabolism of chlorzoxazone. Chem. Res. Toxicol. 6:852857, 1993.

218. K. Nagata, T. Matsunaga, J. Gillette, H. V. Gelboin, and F. J. Gonzalez. Rat testosterone 7-alpha-hydroxylase: Isolation, sequence, and expression of cDNA and its developmental regulation and induction by 3-methylcholanthrene. J. Biol. Chem. 262:27872793, 1987.

219. S. M. Black, S. Ellard, R. R. Meehan, J. M. Parry, M. Adesnik, J. D. Beggs, and C. R. Wolf. The expression of cytochrome P450IIB1 in *Saccharomyces cerevisiae*

results in an increased mutation frequency when exposed to cyclophosphamide. Carcinogenesis 10:21392143, 1989.

220. B. Zurbriggen, E. Böhlen, D. Sanglard, O. Käppeli, and F. Fiechter. Controlled expression of heterologous cytochrome P450e cDNA in *Saccharomyces cerevisiae.* I. Construction and expression of a complete rat cytochrome P450e cDNA. J. Biotechnol. 9:255272, 1989.

221. K. M. Kedzie, R. M. Philpot, and J. R. Halpert. Functional expression of mammalian cytochromes P450IIB in the yeast *Saccharomyces cerevisiae.* Arch. Biochem. Biophys. 291:176186, 1991.

222. Y. Imai. Characterization of rabbit liver liver cytochrome P-450 (Laurate w-1 hydroxylase) synthesized in transformed yeast cells. J. Biochem. 103:143148, 1988.

223. W. R. Brian, P. K. Srivastava, D. R. Umbenhauer, R. S. Lloyd, and F. P. Guengerich. Expression of a human liver cytochrome P-450 protein with tolbutamide hydroxylase activity in *Saccharomyces cerevisiae.* Biochemistry 28:49934999, 1989.

224. T. Uno, and Y. Imai. Identification of regions functioning in substrate interaction of rabbit liver cytochrome P-450 (laurate (omega-1)-hydroxylase. J. Biochem. 106:569574, 1989.

225. R. Kato, T. Yasumori, and Y. Yamazoe. Characterization of human P450IIC isozymes by using yeast expression system. Methods Enzymol. 206:183190, 1991.

226. M. B. Faletto, P. Linko, and J. A. Goldstein. A single amino acid mutation (Ser_{180}>>Cys) determines the polymorphism in cytochrome P450g (P4502C13) by altering protein stability. J. Biol. Chem. 267:20322037, 1992.

227. T. Uno, and Y. Imai. Further studies on chimeric P450 2C2/2C14 having testosterone 16b-hydroxylase activity which is absent in the parental P450s. J. Biochem. (Tokyo) 112:155162, 1992.

228. M. P. Lopez Garcia, P. M. Dansette, P. Valadon, C. Amar, P. H. Beaune, F. P. Guengerich, and D. Mansuy. Human-liver cytochromes P-450 expressed in yeast as tools for reactive-metabolite formation studiesoxidative activation of tienilic acid by cytochromes P-450 2C9 and 2C10. Eur. J. Biochem. 213:223232, 1993.

229. M. P. López-Garcia, P. M. Dansette, and D. Mansuy. Thiopene derivatives as new mechanism-based inhibitors of cytochromes P-450: Inactivation of yeast-expressed human liver cytochrome P-450 2C9 by tienilic acid. Biochemistry 33:166175, 1994.

230. T. Sakaki, M. Shibata, Y. Yabusaki, H. Murakami, and H. Ohkawa. Expression of bovine cytochrome P450c17 cDNA in *Saccharomyces cerevisiae.* DNA 8:409418, 1989.

231. M. Shibata, T. Sakaki, Y. Yabusaki, H. Murakami, and H. Ohkawa. Genetically engineered P450 monooxygenases: Construction of bovine P450c17/yeast reductase fused enzymes. DNA Cell Biol. 9:2736, 1990.

232. N. Yokotani, R. Bernhardt, K. Sogawa, E. Kusunose, O. Gotoh, M. Kusunose, and Y. Fujii-Kuriyama. Two forms of w-hydroxylase toward prostaglandin A and laurate. J. Biol. Chem. 264:2166521669, 1989.

233. W. R. Brian, M.-A. Sari, M. Iwasaki, T. Shimada, L. S. Kaminsky, and F. P. Guengerich. Catalytic activities of human liver cytochrome P-450 IIIA4 expressed in *Saccharomyces cerevisiae.* Biochemistry 29:1128011292, 1990.

234. J.-P. Renaud, C. Cullin, D. Pompon, P. Beaune, and D. Mansuy. Expression of human liver cytochrome P450 IIIA4 in yeastA functional model for the hepatic enzyme. Eur. J. Biochem. 194:889896, 1990.

235. M.-A. Peyronneau, J.-P. Renaud, G. Truan, P. Urban, D. Pompon, and D. Mansuy. Optimization of yeast-expressed human liver cytochrome P450 3A4 catalytic activities by coexpressing NADPH-cytochrome P450 reductase and cytochrome b5. Eur. J. Biochem. 207:109116, 1992.

236. M.-A. Peyronneau, J.-P. Renaud, M. Jaouen, P. Urban, C. Cullin, D. Pompon, and D. Mansuy. Expression in yeast of three allelic cDNAs coding for human liver P-450 3A4different stabilities, binding properties and catalytic activities of the yeast-produced enzymes. Eur. J. Biochem. 218:355361, 1993.

237. V. S. Fujita, D. J. Thiele, and M. J. Coon. Expression of alcohol-inducible rabbit liver cytochrome P-450 3a (P-450IIE1) in Saccharomyces cerevisiae with the copper-inducible CUP1 promoter. DNA Cell Biol. 9:111118, 1990.

238. J. K. Ritter, I. S. Owens, M. Negishi, K. Nagata, Y. Y. Sheen, J. R. Gillette, and H. A. Sesame. Mouse pulmonary cytochrome P-450 naphthalene hydroxylase: cDNA cloning, sequence, and expression in *Saccharomyces cerevisiae.* Biochemistry 30:1143011437, 1991.

239. E. Y. Krynetsky, V. L. Drutsa, I. E. Kovaleva, V. N. Luzikov, and V. Y. Uvarov. Effects of amino-terminus truncation in human cytochrome P450IID6 on its insertion into the endoplasmic reticulum membrane of *Saccharomyces cerevisiae.* FEBS Lett. 336:8789, 1993.

240. G. T. Tucker, M. S. Lennard, S. W. Ellis, H. F. Woods, A. K. Cho, L. Y. Lin, A. Hiratsuka, D. A. Schmitz, and T. Y. Y. Chu. The demethylenation of methylenedioxymethamphetamine (ecstasy) by debrisoquine hydroxylase (CYP2D6). Biochem. Pharmacol. 47:11511156, 1994.

11
Integration of Pharmacokinetics into Drug Discovery and Development: The Alternative Approaches

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I. Introduction

Many disciplines contribute to the drug discovery and development processes. Some of them, for example, chemistry, phytochemistry, and medicine, have their roots in antiquity. Others, for example, pharmacology, biochemistry, and toxicology, are of more recent origin, while a third group, for example, molecular biology, biometrics, pharmacokinetics, and drug metabolism are more recent newcomers. The degree to which the contribution of these many disciplines can be orchestrated into the drug discovery and development process may determine the overall success and fate of research and development (R & D) in an organization.

Pharmacokinetics and drug metabolism (PDM) is thus a relative newcomer to pharmaceutical R & D. Until quite recently, metabolism science and technology smoldered on the back burner, assuming a role of insignificant importance relative to the more established disciplines of chemistry, pharmacology, and clinical medicine, while pharmacokinetics was almost nonexistent. A new drug application (NDA) submitted in 1960 would contain precious little pharmacokinetic or metabolism data, since these disciplines played only a minor role in preclinical and clinical R & D.

Aggressive advances in analytical technology, and an increasing awareness of the importance of pharmacokinetics and metabolism in all aspects of drug activity, have changed the situation. Scientists and physicians have become aware of the critical importance of PDM characteristics in drug action and interactions, and PDM now plays a central and pivotal role in clinical medicine, and in R & D programs. It is of interest that one of the youngest disciplines in drug development has become one of the most important [1].

The explosive growth in awareness, technology, and impact of PDM in pharmaceutical R & D may be compared to similar events in biotechnology. Both have evolved rapidly during the last 1520 years, and both continue to increase their impact on drug discovery and development to a similar degree, but in different ways. Biotechnology has evolved in a well-defined environment. It belongs to discovery and, together with molecular modeling, is providing a new driving force in discovery that will lead the pharmaceutical industry into the 21st century. Biotechnology then, despite its wide range of scientific activities, has a well-defined place in pharmaceutical R & D.

PDM has not been so fortunate. The rapid emergence of pharmacokinetics and metabolism has at once given those concerned with drug discovery and development great opportunities, but also great problems. The combination of these factors has generated a perplexed environment that surrounds PDM in the industrial environment

today. The question, Where does it belong?, which has been answered readily for biotechnology (indeed no such question really existed), has caused considerable debate and conflict for PDM. Only now that

the importance of these disciplines has become so apparent has some resolution been obtained, and this has been fragmentary and traumatic in some instances. The question of where PDM belongs may be more correctly stated as to whom it belongs. The considerable interactions between PDM and other disciplines, and the critical impact that these may have on the activity and success of R & D programs, have caused debate on ownership and identity. This chapter addresses this problem, examines the alternative philosophies together with organizational structures, and offers some considerations as to the optimal approach.

II. The R & D Sequence

The sequence of events that occurs during a R & D program is described in Figure 1. Although the processes of discovery and development are in a state of continuous change in order to meet evolving scientific and regulatory circumstances, the basic sequence of R & D is relatively constant. Currently, the major focus of R & D managers is to minimize the time interval between discovery and NDA submission, and also the costs involved.

PDM contributions to the entire process are often considered separately under discovery and development. However from a PDM perspective, a true distinction between these segments of the program does not exist. Indeed, such a distinction probably does not exist for any discipline when one appreciates that the whole process of discovery and development is a dynamic continuum rather than a series of discrete events.

A. Discovery

Discovery of new chemical entities (NCEs) has traditionally been the prerogative of chemists, pharmacologists, biochemists, and, recently, molecular biology and computer-assisted drug design. Clinical colleagues may have played an advisory

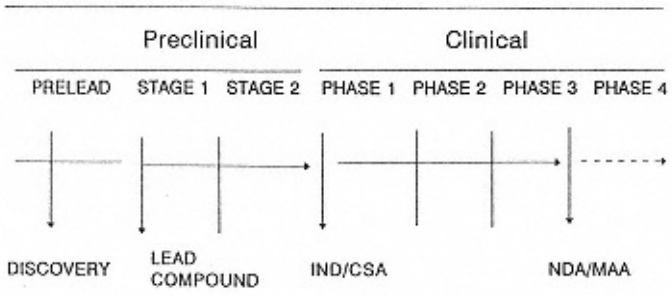

Figure 1
Preclinical and clinical drug development.

role, while input from toxicology and PDM was essentially nonexistent. Little or no pharmacokinetic or metabolism information was available, or requested, until after a compound had entered early or exploratory development.

This situation has changed dramatically, at least from a PDM perspective. PDM data are now requested (demanded!) during early discovery. The major impetus for increased demands for PDM involvement during discovery has resulted from rapid advances in metabolism technology, including in vitro screening [2]. Early incorporation of metabolism expertise is sought to provide early characterization of metabolic profiles, or metabolism fingerprinting, to obtain rapid identification of metabolites in order to characterize the chemical basis for drug activity, and also to provide guidance in the synthesis of new chemical leads in a similar or different therapeutic class. The spectrum of uses for metabolism information during discovery is summarized in Table 1.

The intense involvement of metabolism expertise in discovery programs provides an ideal opportunity to rapidly build a metabolism data base. A variety of such data bases is available commercially. It also presents a situation in which metabolism scientists become intimately acquainted with the chemistry and metabolism of compounds in a chemical class, even before compounds enter early development.

Pharmacokinetics is also becoming heavily involved during drug discovery. This has become more prevalent, not so much as a result of rapidly evolving technology or analytical technology, but largely as a result of simple common sense and realization, on the part of pharmacology and chemistry, of the added value that may be derived from early absorption and bioavailability data and characterization of concentration-effect relationships. A new chemical entity will rarely enter early development without some information regarding metabolism and pharmacokinetics. Thus a drug entering development will probably be characterized from PDM perspectives as well as from those of classical chemical and pharmacology perspectives.

Table 1 Uses of Metabolism Information During Discovery

Early metabolism information in preclinical species
Input into structure-activity relationship
Feedback to chemistry to guide synthesis of new chemical leads
Feedback to pharmacology regarding possible mechanism and duration of action
Input to toxicology regarding toxicological events and target organ specificity
Input into pharmacokinetics regarding presystemic and systemic hepatic clearance in preclinical species, and in humans

During the discovery period there is increasingly close cooperation between PDM scientists and those in other disciplines. PDM scientists are, or should be, on discovery teams and there is considerable potential for interplay between disciplines. This type of interaction is critical for sharing information and concepts that collectively help the team to better select and advance compounds into the development pipeline. The high level of interdisciplinary interaction involving PDM scientists promotes, and correctly so, a feeling of family or ownership among the team players and provides a compelling argument for the commitment of PDM scientists directly to discovery teams.

During the discovery phase, PDM scientists interact closely with most disciplines associated with drug discovery and also some more commonly associated with early development. Major interactions occur with chemistry, pharmacology, biochemistry, molecular biology, and computer-assisted drug design. Interactions may occur also with analytical chemistry as part of the rapid evolution of analytical methodology for compounds in biological and nonbiological matrices. As indicated earlier, the intensity of interaction between PDM scientists and the major discovery disciplines continues to increase in the form of joint studies and continuous information exchange to increase the accuracy, efficiency, and speed of the discovery process.

B. Exploratory Development

Exploratory development may be considered to represent the period from initial declaration of lead status through phase 1 or early phase 2 clinical trials. This is a period during which a compound evolves from a preclinical environment through the investigational new drug (IND) application, to early clinical safety tolerance and efficacy trials.

Probably the most important event during this period, apart from submission and, it is hoped, acceptance of the IND, is initiation of toxicology programs. Conduct of these studies has evolved rapidly from essentially a stand-alone activity within toxicology to an intimate interface with PDM, as expressed by the new discipline of toxicokinetics. PDM collaboration in this major component of drug development has become essential in order to provide accurate validation of the species involved in terms of the nature and extent of metabolism of the toxicology species relative to humans, the concentration-effect relationships, and also safety margin in terms of concentration rather than dose. Thus the interaction between PDM and toxicology scientists continues throughout the entire toxicology program, which extends from the preclinical stage through clinical phase 2B, and often into clinical phase 3 [2].

The exact nature of PDM involvement in toxicology studies has been described in detail

elsewhere [3]. It may be considered to be a formal extension of discovery studies to provide complete characterization of the metabolism and pharmacokinetics of a compound at toxicological doses in toxicology species,

validating the ability of the toxicology and safety data to predict the situation in humans. PDM may be instrumental in selecting one toxicology species over another, especially in terms of metabolism profiles obtained between species relative to humans [4].

Typical PDM studies during exploratory development are described in Table 2. Information is obtained on a building block principle. Each study or series of studies builds on or borrows from earlier studies. Analytical technologies carried through the program undergo incremental improvements in quality, sensitivity, the spectrum of biological fluids handled, validation, robustness, and speed.

Other activities such as autoradiography, drug transport, and bioavailability enhancement round out the spectrum of studies that complete the PDM package for exploratory development.

Almost all of the PDM support studies conducted during exploratory development, whether they be in toxicology, metabolism, pharmacokinetics, or transport, are built on information generated during the discovery phase. Although the discovery and development aspects may be separated in time, they nonetheless represent a spectrum of continuous activity, each study of investigation building on knowledge obtained previously.

Interactions between PDM and other disciplines during exploratory development shift somewhat from those during the discovery phase. Chemistry, phar-

Table 2 Typical Pharmacokinetic and Metabolism Studies Conducted During Exploratory Development

Analytical development
Bioavailability
Biopharmaceutical profile
Single and multiple dose pharmacokinetics
Dose proportionality
Mass balance
Autoradiography
Toxicokinetics, acute, rising dose, acute, 4-week, 13-week
Metabolite finger printing and major metabolite identification
Enzyme induction
Placental transfer
Secretion into milk
IND summary document preparation
Phase 1 clinical single rising dose tolerance

Phase 1 clinical multiple dose tolerance
Food-drug interactions
Pilot bioavailability

macology, and molecular biology play a reduced although still significant role while interactions with toxicology, clinical pharmacology, and clinical research increase. PDM scientists necessarily serve on all early exploratory development teams, as in the case with discovery teams, so there is considerable ownership of PDM resources by development team members.

The pivotal nature of team interactions, and the need to present data quickly and accurately during the early exploratory development phase, requires that PDM scientists enjoy maximum information flow from the discovery phase so that they are able to link concepts to provide optimum support for exploratory development. This requires very close interaction between PDM discovery and development scientists.

C. Full-Scale Development

Full-scale development may be considered as the period from phase 2, when the drug has been shown to have activity without serious tolerance problems, until submission of the NDA. The spectrum of PDM activity during this period is described in Table 3. This period is dominated by large-scale clinical efficacy studies, studies in patient populations, drug interaction studies, and bioavailability studies comparing clinical and market image formulation lots. All of these

Table 3 Typical Pharmacokinetic and Metabolism Studies Conducted During Full-Scale Clinical Development

Single-dose pharmacokinetics
Multiple-dose pharmacokinetics
Metabolism
Additional fed-fasting studies
Enzyme induction
Mass balance
Formulation bioavailability
Pharmacokinetics comparison with competitive products
Special populations, renal, liver disease
Elderly vs. young
Drug-drug interactions
Continued toxicology monitoring and toxicokinetics, long-term toxicology
Therapeutic drug monitoring
Population pharmacokinetics
Pharmacokinetic-pharmacodynamic relationships
Bioavailability/bioequivalence of market image dosage form
Regulatory summary documents

studies carry a heavy PDM component. In fact, many of them are purely pharmacokinetic studies, for example, bioavailability and drug interactions. The technologies associated with these studies build on those used during early development. The observations, particularly on drug-drug interactions, drug-food interactions, and special populations, may require application of concepts and data derived during both the discovery and development phases. For example, an interaction may be revealed in terms of protein-binding displacement or metabolic interference. Interpretation of such data requires knowledge of the absorption, distribution, metabolism, and excretion of a compound, characterized in earlier preclinical and/or clinical studies.

A major proportion of PDM activities during full-scale development, and one that appears to be increasingly emphasized by regulatory agencies, is that of establishing, wherever possible, definitive concentration-effect relationships and the application of mixed effect modeling concepts in order to achieve this [5,6]. Carrying out such analyses require a high level of interaction between PDM and clinical scientists, often with considerable input from biometricians. Carrying out such analyses requires not only sound knowledge of the pharmacostatistical basis of mixed effect modeling but also a clear understanding of the clinical pharmacology of a compound, or class of compounds, in order to establish accurate and meaningful pharmacokinetic-pharmacodynamic relationships. These studies thus require a high level of prospective collaboration between scientists from different disciplines, in order to integrate pharmacokinetic and pharmacodynamic data and establish relationships between these functions in a clinical setting. To this end there needs to be an extensive flow of information from earlier preclinical and clinical studies.

During full-scale development, PDM scientists interact closely with clinical development, clinical pharmacology, biometrics, regulatory, product development, and toxicology. There is a greater degree of interaction than previously since data are systematically accumulated and integrated for final submission of documentation for marketing approval. No one discipline acts alone, but instead as one component of a large number of highly orchestrated and independent activities.

As noted in the case of early development, activities during full-scale development are heavily dependent on information generated during early discovery and development phases, particularly regarding PDM interactive studies with pharmacology and toxicology. These become even more important during the final preparation of the NDA document.

D. The NDA.

Three sections of the NDA are devoted to PDM and PDM-related material. Section 5 contains preclinical pharmacokinetics and metabolism, section 6 contains clinical

pharmacokinetics and metabolism, while section 8 contains clinical

pharmacology data. The toxicology section, also in section 5, contains a considerable and ever-increasing volume of toxicokinetic information, while the clinical efficacy section includes results of pharmacokinetic and pharmacodynamic interaction studies.

Preparation of this volume of documentation requires input from every area of PDM expertise including preclinical and clinical pharmacokinetics and metabolism, distribution, toxicokinetics, and pharmacokinetics/pharmacodynamics. Integration of these data requires close collaboration, not only between PDM scientists and those from other disciplines, but also within the different areas of PDM. For example, clinical pharmacokinetic characterization has to take into account preclinical toxicokinetic safety and metabolism information in order to be meaningful. Description of toxicokinetic data is likewise meaningless unless it relates to clinical pharmacokinetic data and also to metabolism, and particularly the possibilities of species differences in the latter. Description of metabolism data has to include consideration of metabolite kinetics and the relationship between metabolism and metabolism kinetics, and overall activity.

Integration of the different aspects of PDM activities into a final marketing submission document thus requires close collaboration between the scientists involved. The scientists responsible for preparing section 6 have to be familiar with material in section 5, and vice versa. Successful integration of information and data interpretation across sections will result in a reader-friendly document that flows, and tells a story. Failure to achieve the required integration will result in a disjointed document that may not only be reader-unfriendly but may also contain inconsistencies that may need to be laboriously addressed before or after submission of the NDA. Inconsistent and reader-unfriendly data will likely also cause reader fatigue, with consequent delays in approval.

III. Statement of the Problem

The increasing complexity of drug discovery and development and the rapidly expanding role played by PDM in all sections of the process have together created a problem of some dimension. The resource issue is obvious. Demands on PDM resources have become extremely heavy during all stages of discovery and development. If one bears in mind the high attrition rate as compounds progress through the developmental pipeline, careful resource management is required at the discovery end. Most compounds studied at this stage will ultimately die. A second major issue is that of management of PDM resources in order to meet in the most appropriate way the needs of discovery and development teams. These teams naturally seek resource commitment, and covet membership from the various disciplines, including PDM. This frequently leads to competition between teams from discovery and development for PDM resources and also to direct conflict in resource management. How should PDM

be structured and managed in order to meet the many demands fairly and equitably, and provide quality scientific information and support for successful regulatory filing?

IV. The Alternatives

Several alternative approaches to the management and allocation of PDM resources have been used across the pharmaceutical industry. Which alternative is used may be the result of individual preferences within management, lobbying power of particular interests, or influences of other disciplines and structures within an organization. In some horizontally structured organizations (i.e., those divided along therapeutic lines) total resource management may be exercised within a therapeutic area. For example, cardiovascular R & D will have its own team of PDM scientists, committed to and managed by managers of that therapeutic class, as will central nervous system (CNS), chemotherapy, and others. In these cases, PDM resources are administered within each therapeutic area.

A second alternative, or cluster of alternatives, is to administratively distribute PDM resources vertically across the discovery and development pipeline. This may take the form of separate PDM support for discovery and development or for preclinical and clinical, or for discovery, exploratory development, and full-scale development, or combinations of these. In these cases PDM resources are separately administered within segments of the PDM pipeline. Resources may thus be committed to each major segment of the pipeline, with flexibility of resource distribution across therapeutic areas and projects within a particular segment of discovery and development. A common structural alternative is one that separates preclinical discovery and development from clinical development.

A third alternative is to administer PDM as a single team of scientists with allocation of resources vertically across therapeutic areas and horizontally across the R & D pipeline, managed within PDM. This may be called a monolithic approach in that total PDM resources are centrally administered, with resource allocation and relocation vertically and horizontally maintained flexible (negotiable) by means of matrix administration within the overall PDM structure.

The three alternative PDM structures are summarized in Table 4, and are illustrated in Figure 2.

V. Comparison of Alternatives

It is instructive to examine the advantages and disadvantages of the three alternative PDM structures, both from the perspective of the quality of support that each can provide to a R & D program and from that of professional satisfaction of PDM scientists.

Table 4 Three Alternative Administrative Structures for Pharmacokinetics and Drug Metabolism

1. Horizontal: PDM activities administered within therapeutic areas
2. Vertical: PDM activities administered within specific regions of R & D pipeline
3. Monolithic: PDM activities administered as a single unit with resource allocation vertically across therapeutic areas and horizontally across R & D pipeline by internal matrix administration

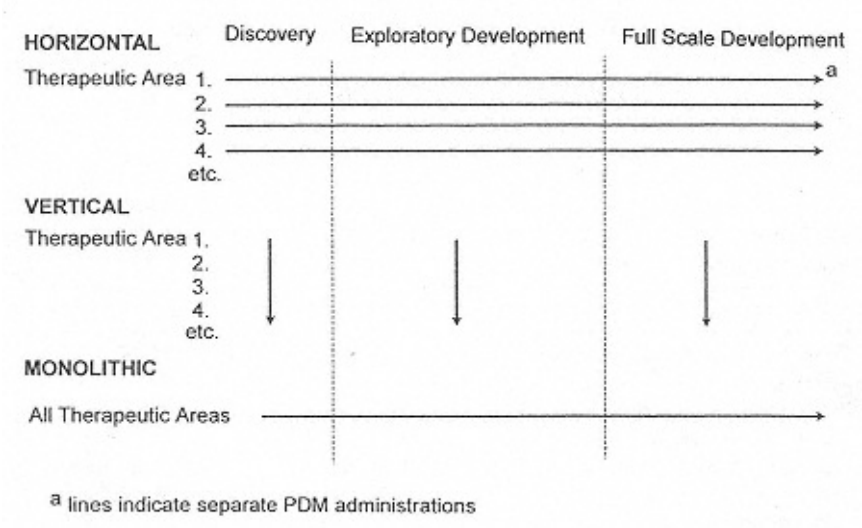

Figure 2
Horizontal, vertical, and monolithic approaches to PDM administration in R & D.

A. Alternative One: Horizontal Administration

With this alternative, separate PDM resources are committed to each therapeutic area. Thus a group of pharmacokinetists and metabolism scientists is separately administered to support a single therapeutic program, for example, cardiovascular. The main advantage of this approach is that the managers responsible for that program have control over PDM resources associated with that program. This could apply to the entire spectrum of activity within an area, from discovery to NDA documentation, or may be subdivided vertically within that area to give a subclass of this alternative (i.e., subdivision of cardiovascular support into preclinical and clinical). Whether subdivided or not, the therapeutic program would have a team of PDM resources committed to and administered within the therapeutic area, thus ensuring PDM support. The therapeutic manager or managers would administer PDM activities to ensure appropriate allocation of resources between projects. The PDM scientists involved would have intimate knowledge of a therapeutic area and of the associated drug candidates and chemical classes. There would be close links (family) between PDM scientists and other disciplines supporting each area and there would be the administrative commitment necessary for effective team support.

The disadvantages of this alternative, whether subdivided along the discovery and development pipeline or not, reside in the fragmentation of PDM resources, loss of critical mass, and loss of flexibility in PDM support from one therapeutic area to another. In addition, there is a high probability of minimal exchange of information between PDM groups reporting to different therapeutic areas unless particular efforts are made to ensure that this occurs. The advantages and disadvantages of the horizontal alternative are summarized in Table 5.

Table 5 Advantages and Disadvantages of the Horizontal Alternative for PDM Administration in R & D

Advantages	Disadvantages
Control of PDM resources within a therapeutic area	Fragmentation of overall PDM resources
PDM scientists have close familiarity with a particular therapeutic area	Lack of critical mass
Close links between PDM scientists and other disciplines supporting each therapeutic area	Minimal information exchange among PDM scientists supporting different therapeutic areas
Administrative commitment for effective therapeutic team support	

B. Alternative Two: Vertical Administration

With this alternative, separate PDM resources are administratively committed to different segments of the discovery and development pipeline. Thus there may be two or three distinct PDM groups, each supporting a different phase of R & D, with compounds being handed from one group to another as they move through the pipeline.

This type of PDM activity may be technically dissimilar from one segment of the pipeline to another. As indicated earlier, the discovery phase requires extensive analytical and in vitro and in vivo metabolism in addition to pharmacokinetic input. Early development requires extensive toxicokinetic, metabolism, and pharmacokinetic support, while full-scale development requires input in the areas of clinical pharmacokinetics, pharmacodynamics, drug interactions, bioequivalence, and pharmacokinetics in disease states and other populations.

The advantages of the vertical approach reside in the commitment of PDM resources to particular phases in the discovery and development pipeline. Thus, the manager of a drug discovery program would administratively manage a group of PDM scientists committed to that phase and would have the flexibility of allocating PDM resources among the therapeutic areas in that phase. Total commitment would thereby enable individuals to focus on a particular segment of activity, with little dilution of effort across the large number of PDM activities that present as a compound moves through the R & D pipeline. The popular practice of separating preclinical development from clinical development provides the opportunity for PDM scientists to focus on these particular areas, and for the relevant managers to concentrate resources on the different activities associated with these areas.

With this approach, individuals have the opportunity to become specialists in particular phases of R & D. Since PDM groups would be administratively managed within particular areas, (i.e., discovery, preclinical, clinical pharmacokinetics), this would permit preclinical PDM staff to focus on pharmacokinetics in animals, metabolism, and toxicokinetics, and clinical PDM staff to focus on the unique aspects of clinical pharmacokinetics, dose-response, and drug interactions in humans.

The disadvantages of this alternative are generally quite different to those associated with alternative one, although there are some similarities. The major problems faced are those of lack of ownership, restricted exchange of information, reduced efficacy, lack of commitment, possible waste of resources, territory problems, and communication and accountability problems. There may also be loss of professional satisfaction on the part of PDM scientists. Lack of ownership must occur when a compound passes through a

number of hands during its transition through the pipeline. This may be inevitable for some disciplines. For example, discovery chemists tend to have more economic interest

than scientific interest in a compound as it enters the NDA preparation stage, while clinicians tend to have greater interest in phase 3 candidates than in early discovery compounds. However, with some other disciplines, for example, PDM, it is not inevitable and should be avoided whenever possible. Waste of resources may result from inadequate or inefficient transfer of knowledge from one group to another as a compound progresses through the development pipeline. A scientist who is intimately familiar with certain aspects of a compound may or may not pass all this information along and not all nuances of a compound characteristics may be readily available from research reports. Territory concerns and lack of accountability are also potential problems in this situation; excellent interdepartmental team work is required in order to maintain a team spirit across departments in this environment. Maintenance of accountability by the separate PDM groups can become a significant problem. Nobody owns or is prepared to assume total responsibility for the overall PDM program to support a particular compound as it passes from hand to hand through the pipeline.

Professional satisfaction may or may not suffer in the case of alternative two, depending on individual circumstances. A PDM scientist administratively associated with full-scale clinical development may find intense personal satisfaction in being associated with the exciting, if harrowing, final stage of development leading to the NDA. On the other hand, he or she may become frustrated with the lack of control of the design and conduct of pharmacokinetic studies during this period due to the multifactorial nature of the studies and the multidepartmental inputs that are inevitable. PDM scientists working in early development, on the other hand, may obtain great satisfaction from the high level of scientific activity involved during this phase and yet may be frustrated at having to pass a compound on to another PDM administration just when it is really showing promise.

A final and major disadvantage of this approach lies in the preparation of the NDA. As indicated earlier, this is a highly integrated document that combines information from preclinical and clinical PDM, together with information from many other departments, to produce an integrated summary of a compound suitable for submission for marketing authorization. Preparation of PDM components, and integration of these in a meaningful and reader-friendly format, require close collaboration between all aspects of PDM activity, ranging from early stability studies conducted during the discovery phase to the final bioequivalence studies of clinical and market image formulations. At this point close collaboration between scientists from all sections of the PDM support process, and equally close collaboration between them and other preclinical and clinical disciplines, is essential. With alternative two, in which PDM support is fragmented along the R & D pipeline, this type of collaboration and integration

of data is more difficult than with alternative one. Advantages and disadvantages of alternative two are summarized in Table 6.

C. Alternative Three: Monolithic Administration

The third alternative, referred to here as the monolithic approach, differs from the previous two in that all PDM activities, from discovery to the NDA submission, are coordinated under a single administration with horizontal and vertical support being managed by appropriate internal organization, probably in form of a team matrix structure. With this approach close collaboration is maintained between PDM scientists and therapeutic teams by a team membership approach and therapeutic areas may be supported by different groups within the PDM organization. As a compound progresses from the discovery phase into and through the development pipeline toward the NDA, it evolves through appropriate groups within PDM, or may continue with the same group depending on the appropriate support structure for that compound or therapeutic class. The advantages of this alternative are severalfold. They include focused and coordinated PDM management, economy of resources for operating and capital budgets, little or no duplication of effort, ownership and accountability in a

Table 6 Advantages and Disadvantages of the Vertical Alternative for PDM Administration in R & D

Advantages	Disadvantages
PDM resources administratively committed to particular phases of discovery and development pipeline	Fragmentation of PDM resources Loss of critical mass Loss of flexibility for PDM support along R & D pipeline
Flexibility of resource allocation among therapeutic areas within a particular phase of the pipeline	Lack of commitment to therapeutic areas
PDM scientists able to focus on a particular segment of discovery and/or development	Potential waste of resources Difficult and possibly inefficient information and technology transfer between separate PDM groups as compounds move through pipeline
No dilution of PDM activity as compounds move along R & D pipeline	Lack of accountability Difficult communication
PDM scientists can specialize in particular aspects of R & D	Lack of professional satisfaction Problems of integrating data and concepts in final regulatory documents

particular compound or therapeutic class, efficient technology transfer, lack of territory problems, and a high critical mass for resource allocation flexibility. With this approach, a single body of PDM scientists is responsible for the support of an entire program of R & D activity while champions for therapeutic areas or development phases do (and should) exist within the PDM organization.

In addition, the third alternative does not promote elitism, which can and often does occur in a fragmented organization. Overall PDM management responsibility would be to support projects equitably across R & D both vertically among therapeutic areas and horizontally along the R & D pipeline. The ability to plan across a broad spectrum of activities enables PDM management to apply resources fairly and to muster resources to focus on particular areas when necessary. The large critical mass available permits ready mobilization of expertise on demand, unencumbered by administration burdens. Economy of management of capital and operating resources is possible because technology transfer is minimal and, in many cases, unnecessary, with no duplication of effort. Since a single department is responsible for all PDM activities, accountability and ownership are mandatory. There is nowhere to pass the buck. These advantages naturally assume a certain quality of flexible and responsive management, and also close collaboration within PDM. These qualities are a must and no organization, no matter how simple or complex, can function efficiently without them.

A single PDM organization, managed effectively, provides an effective environment to support R & D in that flexibility, devoid of administrative barriers, alliances, and misconceptions, exists to support the dynamic events in an R & D program. The large critical mass, composed of a spectrum of analytical, metabolism, and pharmacokinetic technology, can be rapidly mobilized to support important projects and to meet unsuspected (or sometimes suspected but difficult to achieve) deadlines. Through a combination of line and matrix interactions, which are common and necessary in this type of multifaceted organization, rapid sharing or transfer of information technology is possible, unencumbered by obstacles that might occur in the previously discussed alternatives. These factors, together with continued ownership of projects within the department, represent considerable advantages.

The disadvantages of this approach are more difficult to identify. However, if the main advantages of the approaches described in alternatives one and two are those of commitment and close familiarity with a particular therapeutic area or discovery or development phase, lack of such commitment would be a disadvantage with the monolithic PDM structure. This may not necessarily be the case, however, because in the latter structure the extent of commitment should be maintained at a high level and

exercised through the different sections within PDM. The only other possible disadvantage would be the need for PDM to manage its own resources efficiently in order to provide effective support to the spectrum of activities that need its input. To meet this need, PDM management

would need to exercise a total and unbiased commitment to the whole R & D pipeline.

The professional advantages to a PDM scientist working in this monolithic administrative environment are legion. Since all PDM activities, across disciplines, therapeutic areas, and discovery and development phases, are handled, segmentally, under a single administration, there is considerable opportunity for scientists to be exposed to a broad spectrum of activities. Active participation in each area may not occur, and is probably unrealistic. However a high degree of involvement among PDM disciplines to support particular compounds and the broad spectrum of exposure provides a rich and professionally gratifying environment. Together with this comes the concept of pride of ownership and also responsibility. The buck stops here. The advantages and disadvantages of this third alternative are given in Table 7.

Table 7 Advantages and Disadvantages of the Monolithic Approach for PDM Administration in R & D

Advantages	Disadvantages
PDM activities coordinated under a single administration	Necessity for flexible and responsive PDM management
Large critical mass for resource allocation	Possible lack of commitment to particular therapeutic or developmental areas
Flexibility	
Horizontal and vertical support managed by internal matrix structure	
Economy of resources, both operating and capital	
Little or no duplication of effort	
Ownership	
Accountability	
Efficient information and technology transfer	
No elitism	
High degree of professional satisfaction among PDM scientists	

VI. Recommendations and Conclusions

The three alternative approaches for the structure of overall PDM support for drug discovery and development have been presented here in an attempt to tease out the most

important factors identifying each, and to compare their advantages and disadvantages.

The arguments are necessarily generic and do not apply to all R & D situations, or to some particular types of expertise. It would be illogical to fragment such technologies as autoradiography, immunoanalysis, metabolism, mass spectrometry, and other highly specialized and resource-intensive activities. By definition, these should be and generally are centralized within the PDM organization. The arguments likewise do not necessarily apply to small or highly specialized R & D organizations. In many such cases, particularly those engaged in small high-technology focus areas, PDM contribution may be at a minimum or may not begin to play a part until potential new chemical entities enter development, possibly at a different organization.

The arguments are thus limited to the large organizations, the major pharmaceutical houses, with comprehensive discovery and development programs. Within these types of organization the three alternative PDM structures described here are currently being used. Of course there are other alternatives, and also modifications of those considered here. However alternatives one, two, and three represent the great majority of cases. Of these, alternative two may be the most common, largely as a result of separation of metabolism, preclinical, and clinical support, followed in decreasing infrequency by alternatives one and three.

It is difficult to compare the alternatives objectively because every organization has its own internal characteristics and interests. An alternative that fits ideally into one organization may simply not work in another. As with all intraorganizational situations, most schemes will work given good management, good people, and, of course, a productive and successful environment. Also, any structural approach must ultimately benefit the entire organization. An organization that is good for a PDM scientist may or may not be satisfactory for interacting with colleagues in other departments. In the pharmaceutical discovery and development environment of continuous challenge and change, together with resource limitations with little foreseeable relief given the present regulatory environment, organizational structures will also involve compromises and will be subject to stress and challenges. The advantages and disadvantages of the three alternatives must therefore be considered in the light of these qualifying factors.

Given the arguments for the three alternative PDM structures, one can draw certain conclusions. Alternatives one and two may be more satisfactory from a purely local perspective, that is, from the perspective of a manager of a therapeutic program or of a segment of a R & D pipeline. These managers will have control of appropriate PDM resources and can utilize them to their best advantage. The PDM scientists employed within these areas will also become expert in, but not necessarily limited to, these particular areas. On the other hand, these alternatives suffer from the potential problem of

barriers to transfer of knowledge and information, compound unfamiliarity, and also lack of critical mass in many cases for PDM scientists to maintain the flexibility essential in

these types of programs. The overall PDM support program will be under the direction of several managers, which may or may not give rise to problems. Certainly this is unlikely to be optimal for PDM scientists, even if a high degree of specialization is sought by a PDM scientist. These types of fragmented PDM organizations will not be conducive to good collaboration or to good information and technology exchange between PDM scientists in different groups. The overall potential for top quality PDM support for R & D may not be realized.

Alternative three, on the other hand, will undoubtably deprive the therapeutic area or pipeline segment manager of control of PDM support for the area under his or her management. However, from a more global perspective, PDM administrative control would be replaced by healthy competition based on priorities as agreed by R & D upper management and thus an equitable distribution of PDM resources across therapeutic areas and across the R & D pipeline would likely be obtained. Of course, if there were unlimited resources there would be no issue, but this is rarely the case. PDM departments across the industry are invariably understaffed and there is currently much debate within the industry regarding shortcomings in PDM resources to meet regulatory demands, and also to address the question of what spectrum of PDM support is nice to have and what is really necessary to adequately support marketing applications.

Resolution of these questions will take time. In the meantime, PDM departments labor for the most part with inadequate resources to meet demands. The monolithic structure of PDM thus provides central administration for PDM resources, the ability to assign and reassign resources on overall R & D priorities, and flexibility to do this quickly and effectively by maintaining responsibility and also internal familiarity of programs under one roof as a compound moves through the pipeline. The monolithical alternative provides, for the PDM scientist, a superior environment for information and, of equal importance, a broad spectrum of exposures and opportunities ideal for professional satisfaction and advancement.

In addressing the problems of PDM involvement in R & D this writer has come from the perspective of a PDM scientist and administrator. Thus a certain bias is inevitable. However, an attempt has been made to represent and analyze fairly the three major alternatives most commonly employed in the pharmaceutical industrial environment, and to describe the impact these alternatives may have on the efficiency with which PDM may support the activities associated with drug discovery and development, on the professional satisfaction of PDM scientists, and on PDM as a professional discipline which is now central and essential to the drug R & D process.

Regardless of the relative merits of the various methods of incorporating PDM science

into a drug R & D program, each one will work and has worked

in the past provided the program has good management and a collegial atmosphere is established through close collaboration. It is all a matter of teamwork.

References

1. D. B. Campbell and R. M. J. Ings. New approaches to the use of pharmacokinetics in toxicology and drug development. Human Toxicol. 1:469479, 1988.

2. F. de la Iglesia. Perspectives and future directions of toxicokinetics in drug development. In Drug Toxicokinetics. Edited by P.G. Welling and F. de la Iglesia. Marcel Dekker, New York, 1993, pp. 381402.

3. P. G. Welling. Role of pharmacokinetics in drug discovery and development. In Pharmacokinetics, Handbook of Experimental Pharmacology. Edited by P.G. Welling and L. Balant. Springer Verlag, Heidelberg, 1994, in press.

4. M. J. Humphrey and D. A. Smith. Role of metabolism and pharmacokinetic studies in the discovery of new drugs-present and future perspectives. Xenobiotica 22:743755, 1992.

5. L. B. Sheiner and T. M. Ludden. Population pharmacokinetics/pharmacodynamics. Annu. Rev. Pharmacol. 32:185209, 1992.

6. C. C. Peck, W. H. Barr, L. Z. Benet, J. Collins, R. E. Desjardins, D. E. Furst, T. G. Harter, G. Levy, T. Ludden, J. H. Rodman, L. Sanathanan, J. J. Schentag, V. P. Shah, L. B. Sheiner, J. P. Skelly, D. R. Stanski, R. J. Temple, C. T. Viswanathan, J. Weissinger, and A. Yacobi. Opportunities for integration of pharmacokinetics, pharmacodynamics, and toxicokinetics in rational drug development. Pharm. Res. 9:826833, 1992.

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Pharmacokinetics in Drug Discovery and Development: Nonclinical Studies

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I. Introduction.

In the early stage of the drug discovery process, one may have an interesting new chemical that is yet to be proven useful for any medical purpose. Nonetheless, initial pharmacological data and acute toxicological findings have sug-

gested the compound to be sufficiently promising to warrant the great deal of work that will follow. From the moment of decision to pursue to the actual submission of a new drug application (NDA), a tremendous amount of information will be generated concerning the efficacy and safety of the compound. Numerous animal and/or in vitro models may be used to demonstrate the biological activity against the targeted disease. Initially, subchronic (4 week) toxicological studies will be conducted in one rodent and one nonrodent species in order to ensure safety when introducing the compound into human subjects. Following the submission and approval of an investigational new drug application (IND), which begins the clinical phase, additional studies will be performed to evaluate the carcinogenicity, embryotoxicity, as well as specific mechanisms of toxicity in appropriate animal models. Whereas this jumble of activities aims to elucidate what the drug does to the body, it is of equal importance to understand what the body does to the drug so that the pharmacological and toxicological data can be properly interpreted for projection to humans. For this purpose, the discipline of pharmacokinetics has become an integral part of the modern nonclinical drug development program.

Current regulations of the U.S. Food and Drug Administration (FDA) do not state the specific type of nonclinical data required for drug registration. Existing regulatory guidelines also vary considerably between different countries. In order to facilitate the drug evaluation process in a global environment, there is an increasing desire by both the pharmaceutical industry and regulatory authorities for more precise, consistent, and practical guidelines for preclinical toxicology and pharmacokinetic studies. Several recent international conferences have focused on the standardization of preclinical safety and toxicity studies [1,2] and, at the second meeting of the International Conference on Harmonization (ICH) in October, 1993, in Orlando, Florida, the subject of Harmonization of Preclinical Pharmacokinetic Data Necessary to Support the Development of Therapeutic Agents was discussed in some depth. To understand better the rationale of any proposed package of nonclinical pharmacokinetic studies, it would be appropriate first to outline the overall objectives of pharmacokinetic research as applied to nonclinical drug development.

II. Objectives of Nonclinical Pharmacokinetic Studies

A. Support for Pharmacology

It has long been recognized that the intensity and duration of the pharmacological effect of a systemically acting drug are functions not only of the intrinsic activity of the drug but also of its absorption, distribution, metabolism, and

excretion characteristics. Pharmacokinetic data obtained from the pharmacological test species are often useful in the interpretation of drug effects. A typical example is a drug that is active following intravenous administration but is considerably less active after comparable oral doses. Having the appropriate pharmacokinetic information could reveal whether the drug is poorly absorbed to yield subtherapeutic circulating levels or experience presystemic biotransformation to an inactive metabolite. This would provide guidance for subsequent decisions, for example, to improve drug absorption by altering the salt form or formulation, to investigate the possibility of making prodrugs, or to consider a parenteral route of administration.

Many drugs show a direct correlation between drug concentration at the target site and pharmacological effect. For example, present knowledge suggests that the bactericidal action of antibiotics is directly related to the drug levels at the site of infection, and the bactericidal effect is lost when antibiotic levels fall below the minimum inhibitory concentration of the invading organisms. The b blockade induced by sotalol in dogs correlates significantly with the log plasma sotalol concentration [3]. Following an intravenous dose of ^{14}C-labeled bromocriptine, the time course of drug-induced hypothermia in cold-room-acclimatized rats parallels plasma bromocriptine concentrations but not total radioactivity levels, thus confirming the parent drug as the pharmacologically active entity [4].

Any knowledge of the effective blood or plasma concentration in animals can be used to guide later studies in humans as the drug trial progresses into the clinical phase. Specifically, a drug should not be considered inefficacious in humans prior to achieving circulating levels that approach the effective concentrations in the pharmacological species. Thus, potentially valuable therapeutic agents will not fall into unwarranted disrepute because of underdosing.

For drugs with an extravascular site of dynamic action, there is often a more complicated relationship in the time course of plasma levels and the pharmacological effect produced. Numerous methods for the simultaneous modeling of the pharmacokinetics (PK) and pharmacodynamics (PD) of these drugs have been introduced (58). Generally a PK-PD model consists of three submodels [5]: a pharmacokinetic model for characterizing drug absorption and disposition; a link model relating the plasma concentration to the concentration in a hypothetical effect compartment or site of action; a pharmacodynamic model that describes the intensity of the drug effect as a function of the concentration in the effect compartment. Recent advances in computer technology have resulted in a number of sophisticated programs that would greatly facilitate the analysis of pharmacokinetic and pharmacodynamic data using these relatively complex models [9].

This type of analysis could yield useful information if a well-defined pharmacological index can be monitored [10,11].

B. Support for Toxicology

An important function of animal absorption, distribution, metabolism, and excretion (ADME) studies is in the support of preclinical drug safety evaluation. In most subchronic toxicology studies, the drug is administered orally to the test species, often in large doses with no assurance of absorption or dose proportionality. Because the question of absorption or exposure is a crucial parameter in the validation of toxicology studies from both a scientific and a regulatory perspective, it must be carefully addressed for each animal model and the specific mode of administration. The importance of this point is exemplified by the hypocholesterolemic agent probucol. In rhesus monkeys administration of probucol in diets containing large amounts of cholesterol and saturated fat resulted in the death of four of eight animals after several weeks, whereas monkeys maintained on low-fat chow and receiving comparable doses of probucol showed no adverse effects over an 8 year period of continuous drug administration [12]. This difference was associated with a significant increase in absorption and resulting blood levels of probucol by lipids [13], a phenomenon that has also been observed in other species [14].

Determination of pharmacokinetic characteristics during the course of a toxicology study may help to interpret certain toxicologic findings. For example, tissue distribution data may be useful in explaining local toxic manifestations. The occurrence of enzyme inhibition or capacity-limited metabolism may lead to drug accumulation accompanied by toxicity. Pharmacokinetic data obtained from acute or subchronic studies during the pre-IND phase are useful in the selection of a proper dose range for subsequent chronic toxicologic trials.

The importance of the measurement of drug concentrations and exposure during animal toxicology studies, in order to compare with the exposure in humans at therapeutic doses, is now gaining general acceptance [15,16]. Comparison of the ADME data obtained from the species used in toxicology studies with those from humans may enable the rational selection of an appropriate animal model for additional toxicity testing, such as carcinogenicity and teratogenicity studies, that will give a more reliable prediction of human safety. Emphasis is often placed on qualitative similarity in overall metabolic pathways between humans and the test species in order to ensure that both are exposed to the parent drug as well as the same metabolites, any of which may contribute to toxicity. It is also desirable that the selected laboratory species demonstrate absorption and excretion characteristics similar to those of humans.

C. Prediction of Human Pharmacokinetics

Preclinical data on drug absorption and disposition can support and guide informed decisions concerning initial clinical trials. They often are helpful in the design of human ADME studies. For a large variety of drugs, the extent of

absorption is remarkably consistent between different animal species and the human [17]. The bioavailability, which describes the amount of drug reaching the systemic circulation intact, however, may differ among species. The following example concerns the hypolipidemic and antiatherosclerotic agent 58-035, an inhibitor of acylcoenzyme A-cholesterol acyltransferase [18]. Compound 58-035 is strongly lipophilic and virtually insoluble in water. In the rat and the dog, gastrointestinal absorption of 58-035 is relatively low, approximately 20%, when the dose is administered in corn oil [19]. Since the oral absorption of this type of compound is known to be affected by administration in lipid vehicles [20], it is possible that a similar lipid effect may apply to the absorption of 58-035 in humans. Therefore, in the subsequent human single-dose ADME study, a group of subjects was included to receive oral doses of 58-035 immediately following a high-fat meal, in addition to others who were given the drug on an empty stomach [21]. The results indicated that compound 58-035 was poorly absorbed in the fasting state, and administration with the high-fat meal significantly increased the extent of absorption (by nearly 10-fold) to 15% of the dose. Indeed, in those subjects who received [3H]58-035 in the fasting condition, no measurable radioactivity levels were observed in blood at any time. Thus, careful planning with the help of animal data in this case not only served as a pharmacokinetic bridge by confirming an important absorption characteristic in humans but also aided in interpreting the results of a costly human study that otherwise would have been difficult.

In contrast, the distribution and elimination characteristics of a drug tend to be more variable between species. For example, although the corticosteroid prednisolone is efficiently absorbed in both the dog and humans, its elimination half-life in the human (approximately 3 h) is nearly three times that observed in the dog [2224]. Marked differences between species were also found in the serum protein binding of prednisolone [25]. Whereas the percentage of prednisolone bound in the dog (~ 65%) and rat (~ 40%) was almost independent of serum drug concentration, the binding in human serum decreased from approximately 90% to 45% as the concentration of prednisolone increased from 0.024 μM to 16 μM.

A great deal of the interspecies variations in such important pharmacokinetic parameters as absolute bioavailability, clearance, and elimination half-life may be attributed to the vast qualitative and quantitative differences in the metabolism of a drug by the host organism [26]. As a result, direct extrapolation of animal disposition data to humans may not be appropriate. This does not imply, however, that pharmacokinetic data obtained in animals cannot be mathematically manipulated to derive estimates of human pharmacokinetic parameters. As mammals, humans share with other mammalian species many similarities in anatomy, physiology, and biochemistry, which together form the

basis of animal scale-up [27]. Many anatomical and physiological variables can be correlated

among mammals as exponential functions of body weight, using the simple heterogonic equation [28]

$$Y = aW^b \quad (1)$$

or its logarithmic transformation

$$\log Y = \log a + b \cdot \log W \quad (2)$$

where Y is a physiological parameter, W is body weight, a is the allometric coefficient, and b is the allometric exponent. A few examples are shown in Table 1. It appears that the anatomical variables are nearly proportional to body weight but the physiological processes tend to vary as the 0.70.8 power of body weight. Thus, physiological function per unit of body weight or per unit of organ weight generally decreases as body size increases. In order to use a specific animal as a model for a physiological or pharmacokinetic parameter, some sort of appropriate time scaling is required [29]. Because the smaller species have greater clearance values (ml/min/kg body weight), chronological time in these species must be decelerated relative to the larger species. The concept of physiological or pharmacokinetic time, in which a physiological or pharmacokinetic event becomes the independent variable, has been discussed in detail [30]. By using a biological clock that measured physiological time (heartbeats) rather than chronological time (minutes), Mordenti [31] demonstrated that ceftizoxime half-life was identical in five mammals. Thus, 50% of a dose of ceftizoxime was eliminated during approximately 7300 heartbeats regardless of the animal species. Similar interspecies correlations have been developed for a variety of drugs

Table 1 Relationship Between Physiological Properties (Y) and Body Weight (W) Among Mammals: $Y = aW^b$

Property Y	Exponent b
Creatinine clearance (ml/h)	0.69
Inulin clearance (ml/h)	0.77
PAH clearance (ml/h)	0.80
Basal oxygen consumption (ml STP/h)	0.73
Ventilation rate (ml/h)	0.74
Weight (g)	
Kidneys	0.85
Heart	0.98
Lungs	0.99
Liver	0.87
Stomach and intestines	0.94
Blood	0.99

Source: Data from Reference 28.

[3234], and interspecies scaling of pharmacokinetics has been the topic of several reviews [35,36]. By generating the values of a pharmacokinetic parameter Y in three or four preclinical species, the value of Y in humans can be projected using Equation (2).

A different approach to projecting animal data to humans is by means of physiologically based pharmacokinetic (PBPK) models [37]. With physiological models pharmacokinetic interpretation is based on known anatomical or physiological values (Table 2 [3840]) and biochemical properties of the drug. Drug concentrations in blood and various tissues are predicted by organ or tissue size, blood flow rates through organs or tissues, drug protein binding, and the relative affinity of drug for blood and the various tissues and organs [41]. Experimentally determined blood and tissue concentration data, normally in small animals, are not fitted to the model but instead are used to verify the predictions.

Table 2 Physiological Parameters in Several Species

Parameter	Mouse	Rat	Rabbit	Monkey	Dog	Human
Body weight (g)	22	500	2330	5000	12,000	70,000
Volume (ml)						
Blood	1.7	22.5a	__	367	670b	5400
Plasma	1.0	19.6	70	220	500	3000
Muscle	10	245	1350	2500	5530	35,000
Kidneys	0.34	3.7	15	30	60	280
Liver	1.3	19.6	100	135	480	1350
Gut	1.5	11.3	120	230	480	2100
Heart	0.095	1.2	6	17	120	300
Lungs	0.12	2.1	17	__	120	__
Spleen	0.1	1.3	1	__	36	160
Plasma flow rate (ml/min)						
Plasma	4.4	84.6	520	379	512	3670
Muscle	0.5	22.4	155	50	138	420
Kidneys	0.8	12.8	80	74	90	700
Liver	1.1	4.7	177	92	60	800
Gut	0.9	14.6	111	75	82	700
Heart	0.28	1.6	16	65	60	150
Lungs	4.4	2.3	520	__	512	__
Spleen	0.05	0.95	9	__	14	240

aBlood volume in 250 g rat.
bBlood volume in 10,000 g dog.
Source: Data from References 3840.

A PBPK model consists of a series of body tissues or organs connected by blood flow in a pattern that approximates the anatomical and physiological reality. An example of this type of model is shown in Figure 1. In a typical setup, the organ in which the drug exerts its pharmacological activity as well as major drug-eliminating organs would be included in the model. When a drug in the bloodstream reaches an organ, it must pass through the capillary membranes into interstitial fluid, and then through the membrane separating interstitial fluid

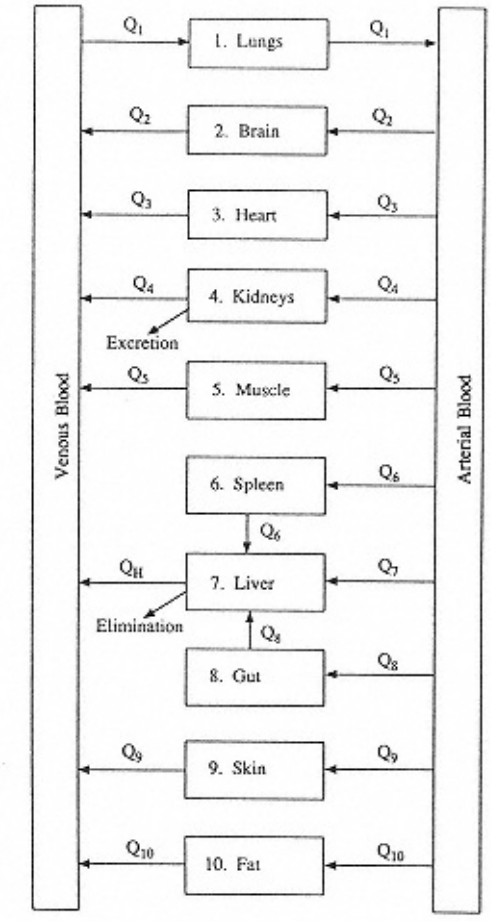

Figure 1
A general physiologically based pharmacokinetic model. Q, blood flow rate to a region; Q^H, hepatic blood flow.

from the cells of that organ, in order to enter the organs cells. For many partially ionized or un-ionized lipophilic compounds, this transport process across membranes is very fast so that drug distribution into a specific organ is limited only by blood flow rate or perfusion into that organ. In PBPK models with perfusion-limited transport, each organ is represented simply by a single compartment. In contrast, for most ionized, hydrophilic compounds membrane transport is relatively slow and rapid equilibrium cannot be assumed. For these compounds, the membrane barrier between interstitial fluid and intracellular space is rate-limiting, thus increasing the complexity and uncertainty of the PBPK model. Nonetheless, when initial modeling efforts assuming simple, perfusion-limited transport fail to yield accurate predictions, improvements often can be achieved by incorporating membrane-limited transport expressions into the model. For example, a PBPK model of cyclosporin has been developed in the rat, assuming that tissue distribution is perfusion-rate limited and that each tissue behaves as a well-stirred compartment [42]. Predictions based on this model tended to underestimate the arterial blood concentrations of cyclosporin during the first 8 h after a rapid intravenous infusion, suggesting that the equilibration between blood and tissues is not rapid enough to be analyzed by an instantaneous equilibrium hypothesis. Reanalysis of the cyclosporin data using a membrane-limited transport model yielded substantially improved agreements between observed and predicted values [43].

A PBPK model that has been validated in an animal species can then be used to predict tissue drug levels in humans that are difficult to obtain experimentally. This information can be useful in guiding clinical drug development. For example, while the human/animal drug concentration ratio in blood may suggest a small safety margin for certain organ toxicity observed in the toxicological species, an estimate of the relative concentrations in the organ in question may reveal a greater safety factor and thus facilitate further clinical trials. However, because of the need of a large data base for model development for each drug, and the relatively complex mathematics associated with the model, this approach has found limited use in preclinical drug development. The physiological model is most useful for elucidating the disposition of drugs for which drug or metabolite concentrations in particular tissues or organs are important (e.g., when target organ for activity or toxicity is well defined). Thus, the PBPK model has been extensively applied to antineoplastic agents [4448] and to quantitative risk assessment for numerous toxicants and carcinogens [4951].

D. Screening New Dosage Forms and Formulations

During the process of developing a final drug product, the formulator develops one or more formulations that demonstrate desirable disintegration and dissolution

characteristics in vitro. The dosage forms are then studied in humans to

evaluate the in vivo release pattern based on the resulting blood level curves. If an adequate blood level profile is obtained, the dosage form is accepted. If no dosage form is acceptable, the entire process has to be repeated. With sophisticated formulation designs such as those used in some controlled-release drug delivery systems, repeated trial and error may be needed before an acceptable product is identified. Such a development process is not only costly but also extremely time-consuming, since a typical human bioavailability study requires the coordination of personnel from pharmacy, drug metabolism, and medical research departments, and often an off-site study center as well. In order to eliminate unnecessary human studies with formulations having potentially sub-optimal in vivo performance, a secondary screening procedure in addition to in vitro dissolution tests can be included prior to clinical trials. One such screen, which is based on simulated blood level-time curves using the product's in vitro dissolution data and the drug's pharmacokinetic parameters in conjunction with a classic pharmacokinetic model, has been described for the development of extended-release oral dosage forms [52]. This process, termed biorelevant dissolution, was successfully applied to the development of a new controlled-release formulation of chlorpheniramine maleate [53]. A more direct approach is to perform in vivo screening tests in animals. Thus, formulations with a desirable release pattern in vitro are submitted for definitive bioavailability or bioequivalence testing in humans only if they also yield a favorable blood level profile in an animal model.

The key to the successful use of animal data in this manner is the selection of a proper model. To this end, it is important to understand the similarities and differences between the anatomy and physiology of the gastrointestinal (GI) tracts of human and the various animal models since they can govern the release of drug from the dosage form, the residence time of the dosage form and released drug at the absorption site, and the permeation of drug across the mucosa. Among the common laboratory species, the rodents and the rabbit are limited by their size, especially in terms of GI dimensions, for studying certain human dosage forms. Swine, on the other hand, have a much larger stomach than the human, and the two also differ in GI transit and intestinal length [54]. Although monkeys are perceived to be closest to humans in terms of evolutionary development and, therefore, response to drugs, their use in bioavailability testing is limited by their availability and also cost associated with special handling and housing requirements. Hence, the beagle dog has become the most commonly used animal species in bioavailability studies for the following reasons:

1. Oral dosage forms intended for humans can generally be administered intact to dogs. However, it is important first to establish dose proportionality, since the resulting dose per unit body weight would be higher in the dog than in the human.

2. The beagle dog is relatively easy to maintain and handle. Its body weight is sufficiently stable over time to allow repeated studies in the same animal, that is, studies using a crossover design. Approximately 100 ml of blood can be withdrawn from a 10 kg beagle every week for 6 weeks without significantly affecting its normal physiology [55].

3. Dog and human share many similarities in gastrointestinal anatomy and physiology [56,57]. Gastric dimensions and GI motility for dog and human are similar, rendering the dog a good model for studying stomach emptying in humans [58]. The luminal diameter in the dog, 2.53.5 cm, is also similar to that in humans.

The suitability of the beagle dog or any other animal model, however, also depends on the specific drug product in question. For example, owing to slightly higher gastric and intestinal pH values in dogs than in humans, the absorption of a poorly soluble drug with a pK_a in the 58 range may differ between the two species [59]. The bioavailability of a drug may show a distinct dependence on gastric acidity in one species but not in the other [60]. The extent of absorption from certain slow-release products may be compromised by the shorter small intestine of the dog (4 m) than that of humans (6.5 m) [61]. In the fasting state, the transit time in the dog is only approximately half that in humans [62,63]. Discrepancies in certain bioavailability parameters between dogs and humans have been attributed to more rapid gastric emptying and transition of drugs in the gastrointestinal tract of the dog [64,65]. Although the dog may differ from the human in the absolute bioavailability of drugs owing to possible differences in first-pass metabolism, it appears to be a good indicator of the rank order of bioavailability of different dosage forms or formulations [6668]. Formulation-related absorption problems in the dog usually also exist in the human [69].

Although bioavailability testing in a suitable animal model can provide useful information on the best choice of formulation for early clinical studies, the definitive bioavailability data should always be obtained in humans. In rare occasions when such studies are not ethically permissible or desirable in humans, for example, for such cytotoxic agents as methotrexate or cyclophosphamide, the FDA has accepted the use of validated animal models for evaluating bioequivalence [70].

III. Types of Studies

A. ADME Studies (Mass Balance Studies)

1. General Considerations

The great majority of ADME studies are conducted to support nonclinical toxicological findings. Therefore, the discussions in this section will focus on this type of study.

a. Animals The species and strain should be the same as those employed in the toxicity study and should be matched as closely as possible with regard to age, weight, and gender. The rat and beagle dog are often employed in preclinical studies since they are the most commonly used species in subchronic toxicity trials. ADME studies in the mouse and rabbit are conducted to support carcinogenicity and embryotoxicity studies, respectively. Other species including the monkey, hamster, and minipig also have been used as test models for certain drugs. The number of animals in each study should be adequate to indicate the variability to be expected within a given species.

b. Drug In order to support toxicological findings, at least two doses encompassing the dosage range used in the subchronic toxicity study normally are tested. These should be prepared in the same dosage form containing the same excipients in identical proportions as in the toxicity study and administered via the same route. An intravenous dose is also usually included to serve as a reference standard. Drug disposition should be studied after a single dose and, preferably, also during multiple dosing.

The conduct of ADME studies can be greatly facilitated by using drug substances appropriately labeled with radioactive isotopes, usually ^{14}C or ^{3}H, since this method provides a simple means of obtaining material balance and allows an estimation of the total exposure to drug-derived material. During the synthesis of radiolabeled compounds, the label should be introduced specifically into a metabolically stable and, in the case of tritium, nonexchangeable position. Computer programs, such as computer-assisted metabolism prediction (CAMP), with a large data base of metabolic transforms, have been found useful in the prediction of biologically stable positions of the compound, thus facilitating selection of the most suitable precursor for the introduction of the label [71]. The in vivo stability of tritium labels can be assessed by preliminary experiments measuring the extent of tritiated water formation. The tritiated water concentration (dpm/ml) in the distillate or lyophylisate of urine samples collected during a designated time interval after dosing, presumably after equilibrium has been reached between urine and the body water pool, is determined. The value is extrapolated from the midpoint of the collection interval to zero time, based on the known half-life of tritiated water in the given species. The percentage of the radioactive dose that is transformed to tritiated water (% $^{3}H_2O$) can be calculated using Equation (3):

$$\% \ {}^{3}H_2O = \frac{[{}^{3}H_2O]_{t=0} \times V}{\text{Dose of Radioactivity}} \times 100\% \qquad (3)$$

where $[^{3}H_2O]_{t=0}$ is the concentration of tritiated water at zero time and V is the exchangeable body water volume. The exchangeable body water content as well as the half-life of tritiated water in some mammalian species are shown in Table 3

Table 3 Volume and Half-Life of Body Water in Some Mammalian Species

Species	Gender	Exchangeable Body Water (% Body Weight)	Half-Life (Days)
Mouse	F	58.5	1.13
Rat	M	59.6	3.53
Rabbit	F	58.4	3.87
Dog	M	66.0	5.14
Cynomolgus monkey	F	64.2	7.23
Rhesus monkey	M	61.6	7.80
Human	M,F	55.3	9.46

Source: Data from References 72 and 73.

[72,73]. The stability of ^{14}C labels, on the other hand, is usually determined by the extent of [^{14}C]carbon dioxide formation.

If the drug is likely or known to fragment into two major portions, it may be advantageous to trace both fragments by providing each with a different label.

The chemical and radiochemical purity of the labeled compound must be verified prior to use. The desired specific activity of the administered radioactive drug obviously depends on the dose to be used as well as the species studied. Doses of ^{14}C of the order of 5 μ Ci/kg for the dog and 20 μ Ci/kg for the rat yield measurable radioactivity levels in blood even when the oral absorption of the drug is relatively poor [19]. Doses of ^{3}H are usually two to three times higher than those of ^{14}C owing to lower counting efficiency associated with the former.

c. Biological Samples Blood, urine, and feces generally are collected from all species; bile and various tissues and organs should be collected at least from the rat and mouse. As an ideal, serial samples should be collected for an adequate duration to define the disposition kinetics of the compound and to achieve mass balance. For the rat and mouse, if the administered dose is not completely recovered, the entire carcass could be homogenized and analyzed for residual radioactivity. Exhaled air is sometimes collected to assess loss of radioactivity in the form of [^{14}C]carbon dioxide.

d. Analysis of Samples Total radioactivity should be measured in all biological samples. When methods are available, the parent drug in blood and selected tissues should also be determined. At the pre-IND stage, metabolite patterns should be established in blood, urine, and bile (or feces), usually by liquid chromatography coupled with radioactivity monitoring [74]. Metabolite isolation, characterization, and identification are usually not

undertaken until

phase I, that is, when the drug appears destined to undergo further development in humans.

2. Rat and Other Rodents

a. Study Design In addition to single oral and intravenous doses, multiple dosing pharmacokinetic characteristics are normally determined after repeated administration of radiolabeled drug for a specified length of time, usually 1 week or longer. The number of rats per dose group and the samples to be collected in a typical ADME study are shown in Table 4. In this example, adequate information on the absorption, distribution, and elimination of the drug would be obtained from 12 rats. Considering a protocol calling for 2 dose levels, single and multiple dosing regimens, as well as a single intravenous reference dose, it would require 60 animals to study either the male or female alone. The total figure is often larger since at least one or two of these studies should be conducted in both sexes in order to identify any gender-related differences in drug absorption and disposition.

b. Dosing In most toxicological studies in the rat, the test drug is administered either by gavage or by incorporation in the diet. Dietary drug administration is usually the preferred mode during chronic toxicity studies because of the convenience of the method and the elimination of trauma and potential danger of pulmonary complications caused by intubation. On the other hand, administration by gavage provides a greater control over the amount of drug delivered and the time of dose and is the method of choice for obtaining pharmacokinetic data. There is now sufficient information in the literature to show that the absorption and/or metabolism of an orally dosed drug may be a function of the mode of administration. Boyd [75] suggested that the administration of the entire daily dose in a single gavage to animals is more likely to

Table 4 Sample Collection from Each Dose Group in a Typical Rat ADME Study

Number of Rats	Samplea
3	2 h tissuesb
3	24 h tissues
3	48 h tissues
3c	Serial blood; urine and feces in 12 or 24 h intervals; tissues at end of study, (e.g., 96 h)

aThe samples are collected at designated times after a single radiolabeled dose or after the final dose during multiple dosing.
bEstimated peak time.
cDuring multiple dosing, predose blood specimens as well as complete

urine and feces for each dose interval are also collected from these rats.

reveal the maximum toxic potential of chronic human dosing than administration of the compound in the diet. However, subsequent studies conducted by various investigators have produced conflicting results. Tse et al. [76] reported that the rate of absorption and the overall bioavailability of the calcium antagonist isradipine were substantially greater from gavage than from diet administration in the rat. The authors attributed the increased bioavailability observed with dosing by gavage to the more rapid presentation of isradipine to the liver, which could have led to partial saturation of presystemic metabolism. As an alternative, it is also possible that the actual amount of isradipine absorbed from the diet was smaller than that from the gavage dose due to potential drug-diet interactions or binding. Although the absorption and bioavailability of the antihypertensive agent captopril were greater after gavage doses than after the same doses given in the diet [77], continuous dietary administration of cefatrizine, a cephalosporin antibiotic, yielded higher, albeit later, peak plasma concentrations and greater overall bioavailability than administration of the same daily dose once a day by gavage [78]. In another study [79], the bioavailability of *N*-acetylprocainamide was unaffected when administered in the diet compared with gavage. Therefore, it is apparent that the effect of gavage or dietary administration on drug absorption and bioavailability is dependent on the compound. Smyth et al. [80] showed that the extent of absorption of water-soluble salts of acidic and basic compounds was equivalent from diet and from oral solutions but the availability of a water-insoluble, neutral compound incorporated in the diet was 70% relative to an oral solution of the compound in polyethylene glycol-400. Other factors, such as potential drug-diet interactions or binding, must also be considered. Therefore, in order for the pharmacokinetic data to reflect drug exposure accurately during the toxicity trial, it is important that the same mode of oral administration be used in both studies.

For administration by gavage, the correct amount of drug is suspended or dissolved in an appropriate vehicle to allow a dose volume of about 10 ml/kg body weight. In order to ensure consistency throughout the entire dose range, a suspending agent, such as sodium carboxymethylcellulose (CMC), is often used even if the drug is readily soluble at the low dose level, since higher doses may exceed the solubility limit of the drug. To prepare a drug-diet mixture, the test compound should be incorporated into pulverized diet by geometric dilution [81] using a mortar and pestle. The drug-diet ratio is determined by the intended dose, the rat weight, and the average daily food consumption, which is approximately 20 g for a 250 g rat. Because of the small amount of drug usually required for a comparatively large quantity of food, thorough mixing is important and content uniformity should be confirmed by dose assays prior to starting the experiment. The design of the rat cage should be such that any spillage of the mixture is recovered and not allowed to contaminate the excreta. The actual dose consumed is recorded at the end of

each feeding cycle.

In addition to the oral dose, a single intravenous dose should also be administered as a reference standard for the calculation of oral bioavailability and absorption. The intravenous dose level is often selected to be near the lower end of the oral dose range, although its tolerability should first be established using nonradiolabeled drug. A simple way of intravenous dosing to the rat is by direct injection via the surgically exposed jugular vein [82]. Under anesthesia using 5% vaporized isoflurane in oxygen [83], a small skin incision (ca. 1.5 cm) is made over the pectoral muscle to facilitate dosing and sutured closed after dosing. Although this method requires surgical preparation of the rat, it appears to be one of the more reliable procedures for intravenous drug administration and, unlike most cannulation techniques, imposes no stress, which may affect at least some aspects of normal physiology resulting in altered drug kinetics [8487]. Intraperitoneal injection, commonly used by pharmacologists for the administration of compounds to small animals, is different from intravenous dosing in that drug absorption from the peritoneal cavity is slower [88] and entails passage into the portal circulation, which can result in incomplete systemic bioavailability [89].

c. Data Collection In rat studies, it is generally feasible to monitor concentrations of drug and related materials in blood as well as various tissues and organs. Furthermore, by confining the animals to properly designed cages, mass balance can be achieved since the potential loss of excreta is minimized.

With the development of sensitive analytical methods that require only micro-samples of blood, pharmacokinetic data from individual rats can be obtained by serial sample collection. Several methods using a chronic indwelling catheter to facilitate repeated blood collection have been described [9094], but the animal preparation procedures tend to be elaborate and tedious and are incompatible with prolonged sampling periods in studies involving a large number of animals. Furthermore, physiological changes caused by the prolonged presence of a cannula in the bloodstream could lead to alterations in the pharmacokinetics and metabolism of the test drug, as have been shown for propranolol, antipyrine, and amoxycillin in the rat [9597]. A simple alternative to cannulation is the nonsurgical method of bleeding the rat from the cut end of its tail [98]. The blood can be easily collected by capillary attraction into heparinized micro-pipettes of desired capacity (25250 µl). As an alternative, a small (25-gauge) needle may be used for direct access to the tail vein [99]. Despite relatively low regional blood flow [85,100], the tail vein has been shown to provide valid blood concentration data for a variety of drugs [82,101]. Relatively large volumes of blood (~ 1 ml) can be obtained by jugular or femoral venipuncture from the unanesthetized rat [102], but the procedure requires restraining the animal and tends to be traumatic. The orbital sinus is another commonly used vein for bleeding from the rat [98], although this method requires anesthesia and is not suitable for

repeated sample collection.

Designated tissues and organs are collected from sacrificed rats by standard surgical procedures [103]. Aliquots of homogenized tissues can be combusted in a sampler oxidizer, with capture of the radioactive products in an appropriate absorption medium and incorporation into a scintillation cocktail. As an alternative, the tissue sample may be dissolved in a solubilizing agent before being added to the scintillant for radioactivity counting. It should be noted that certain biological samples yield colored solutions that may produce severe quenching and low counting efficiencies. Such samples need to be decolorized prior to counting. Bleaching agents that have been used for this purpose include hydrogen peroxide, benzoyl peroxide, sodium borohydride, sodium hypochlorite, and ammonium borohydride [104]. Major tissues of interest (i.e., those showing a high radioactivity content) can then be extracted for the isolation and quantitative determination of parent drug and its metabolites.

The introduction of whole-body autoradiography (WBAR) [105] provided an alternative method of examining tissue radioactivity. Based on the photoradiographic localization of a radiolabeled compound in sagittal sections of the whole animal prepared at appropriate times postdose, a qualitative pattern of drug distribution and accumulation in body tissues and fluids is visualized [106]. The technique is useful in identifying organ or tissue structures with heretofore unsuspected, high concentrations of drug or metabolites. The isotope concentrations in various regions of the body section can then be quantitatively determined by comparative densitometry [107110]. More recently, alternative methods such as storage phosphor technology (SPT) and multiwire proportional counting (MWPC) have been developed for the quantitative analysis of tissue concentrations in freeze-dried sections. Of the three methods, SPT, also known as autoradioluminography, appears to have the greatest potential, offering an acceptable optical resolution, a broad dynamic range, and a sensitivity (speed) approximately 25 times higher than film autoradiography. Developed by Dr. Georges Charpak (Nobel Prize in Physics, 1992), MWPC permits instant counting and imaging of the distribution of beta and gamma activity on tissue slices and other flat samples. Despite its greater speed compared with phosphor screens, however, MWPC produces a relatively low optical resolution and therefore may be inappropriate for the accurate measurement of tissue radioactivity levels. The interested reader is referred to Chapter 8 for an in-depth discussion of the different methods of quantitative autoradiography.

Quantitative collection and analysis of urine and feces at designated intervals postdose can provide useful information on the rate and extent of excretion of drug and/or metabolites via the renal and biliary pathways. Biliary excretion can also be studied directly by surgically exposing the common bile duct and inserting a cannula for bile collection [111]. A recovery period of at least 24 h should be allowed before

administering the test drug in order to avoid possible delays in gastrointestinal transit associated with postoperative adynamic ileus

[112]. Since the loss of bile components may lead to impaired intestinal absorption, particularly for highly lipophilic drugs [113119], a means of bile replenishment is usually needed in this type of experiment [120]. Furthermore, prolonged depletion of bile can result in a 1020-fold increase in bile salt synthesis and a decrease in biliary volume [121]. These changes occur within 12 weeks of bile duct cannulation and could influence the biliary excretion and recycling of drugs. However, in experiments of relatively short duration (< 1 day), the loss of bile appears to have little effect on the biliary excretion of xenobiotics [122].

A drug that is excreted in the bile enters the intestine, where some or all of the excreted dose may be reabsorbed to complete an enterohepatic cycle. Biliary secretion and intestinal reabsorption may continue until the drug is ultimately eliminated from the body via renal or fecal excretion or any other routes of disposition. Hence, enterohepatic circulation may result in unusually long persistence of drug in the body and may influence such pharmacokinetic parameters as the terminal half-life, the area under the blood level versus time curve (AUC), and possibly estimates of bioavailability [123128]. A direct approach to examining the effect of enterohepatic circulation on drug pharmacokinetics quantitatively is to monitor the biliary excretion and intestinal reabsorption in a cascade fashion [129132]. Thus, the bile obtained from a drug-treated, donor animal is administered intraduodenally to a recipient animal, also with a bile fistula, and the excreta from both animals are collected and assayed. Tse et al. [133] described an improved method using a pair of bile duct-duodenum cannula-linked rats. The method permits a realistic approximation of biliary excretion and reabsorption in intact animals and has been applied successfully to the evaluation of enterohepatic circulation of temazepam and metabolites in the rat [134].

For ^{14}C-labeled drugs, the tracer carbon may be incorporated in vivo into carbon dioxide, a potential metabolic product. Therefore, it may be useful to monitor the radioactivity in expired air if analyses of urine and feces fail to yield complete recovery of the tracer dose. Following drug administration, the rat is placed in a special metabolism cage through which room air is drawn by a vacuum pump. Exhaled breath exiting the metabolism cage is passed through an appropriate trapping solution, such as a mixture of 2-ethoxyethanol and 2-aminoethanol (2:1), which captures the expired $^{14}CO_2$ [135]. The trapping solution is replaced and assayed at designated times postdose so that the total amount of radioactivity expired as labeled carbon dioxide can be determined.

3. Dog and Other Nonrodents.

a. Study Design Normally, only single oral and intravenous doses of radio-labeled drug are tested in ADME studies in the dog. Data generated from these experiments allow the

estimation of absolute bioavailability (of parent drug) and overall absorption (of radioactivity) as well as describe the elimination pattern

of the administered dose. Typically three animals are used in each dose group and, because of the relatively long washout period often associated with residual radioactivity, a crossover design is not usually employed. Supplementary experiments requiring specific surgical procedures are sometimes conducted. For example, the common bile duct of the dog and the gallbladder of the monkey can be cannulated for the collection of bile in biliary excretion studies.

b. Dosing The same high and low oral doses as well as the same dosage form used in the subchronic toxicity study should be chosen for the ADME study. If a solid dosage form is selected, it is important that the radiolabeled substance be identical to the nonradioactive drug used in the toxicity study with respect to crystal form and particle size. These factors may have a significant influence on the dissolution rate of the product, which in turn may govern the extent of absorption or bioavailability, especially for poorly soluble drugs [136138]. In general, drug absorption is inversely related to particle size when dissolution is rate-limiting [139143]. For example, the bioavailability of phenytoin from an encapsulated dose in the dog decreased by a factor of about 3 as the particle size increased from 032 to 100 μm [144].

The radiolabeled dose should be administered to dogs under the same condition, fasting or postprandial, as in the toxicity study. Depending on the drug, coadministration of food or fluids may profoundly influence the absorption of oral medications in the dog [145,146], as it does in humans [147151].

A side effect frequently associated with the administration of large oral doses to dogs is the induction of emesis. Therefore, prior testing using nonradioactive drug in separate animals is advisable. In extreme cases, it may be necessary to reduce the dose to a more tolerable level in order to obtain data from a sufficient number of dogs.

c. Data Collection Serial blood samples can be obtained from the dog by direct venipuncture of the cephalic or femoral veins. Complete urine and feces are collected at designated intervals for a sufficiently long period to recover the administered radioactivity, usually 45 days. Fecal samples can be lyophilized and pulverized, or they can be homogenized while wet, and aliquots used for analysis.

Bile is collected when necessary from dogs and other nonrodents with cannulated bile ducts [152155]. Marshall et al. [152] described a chronic bile duct cannulation procedure that permits the bile to exteriorize for collection during an experiment but to drain into the intestine between experiments. The loss of bile over a short collection period (1 day) appears to have little or no effect on bile production in the dog [152], which is similar in this respect to the rat [122]. As shown in Figure 2, the rate of bile flow in four dogs

averaged 4.2 and 4.3 ml/h, respectively, with and without replacement of bile into the duodenum [152].

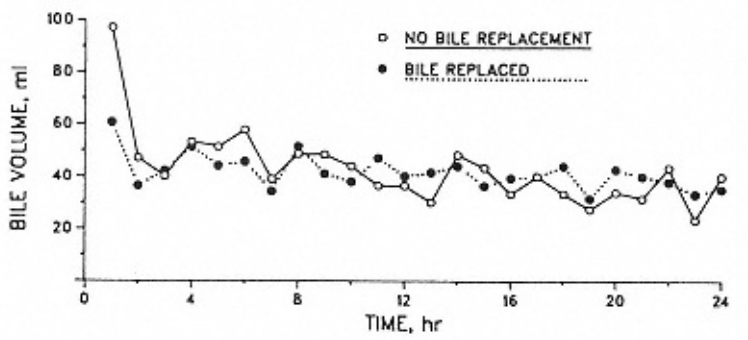

Figure 2
Average bile (hourly) collection from four dogs over a 24 h period studied with and without bile replacements with 1 week between experiments. (Reproduced with permission from Ref. 152.)

Exhaled $^{14}CO_2$ can be collected by restraining the dog in a sling with an airtight plastic cover over its head. The plastic cover should have separate intake and exhaust ports, allowing the animal to breathe normally while simultaneously permitting the quantitative collection of expired air. Exhaled breath is passed through a series of traps in which radiolabeled carbon dioxide is captured.

4. Interpretation of ADME Data

a. Absorption and Bioavailability In the framework of the present discussion, absorption is defined as the process by which a compound and its metabolites are transferred from the gastrointestinal tract (or another site of application) to the systemic circulation. Thus, the absorption of a dose in a given animal reflects the total exposure of the animal to the parent drug as well as all drug-related materials. In contrast, the bioavailability of a drug concerns only the rate and extent to which the administered drug reaches the systemic circulation intact or as the active ingredient. With the exception of some highly lipophilic compounds that are preferentially absorbed via the intestinal lymphatic system [156158], drugs absorbed from an oral dose must pass through the hepatoportal system and, therefore, could be subject to metabolism in the liver before entering the general circulation. Other potential sources of difference between absorption and bioavailability (i.e., first-pass effect) include chemical degradation or metabolism in the gastrointestinal tract and gut wall metabolism.

The absolute bioavailability of a drug or drug product can be estimated by comparison of the resulting plasma drug concentrations or cumulative urinary excretion to those obtained from an intravenous reference dose. The extent of bioavailability is often reflected by the area under the plasma level versus time curve; the rate is indicated by the peak concentration C_{max} and the time to peak

t_{max} following drug administration. Plasma concentration data can also be evaluated by statistical moment analysis in terms of mean residence time (MRT) and variance of residence time (VRT) [159]. These values, which are the first normal and second central moments of the plasma drug profile, respectively, are defined by Equations (4) and (5):

$$MRT = \frac{\int_0^\infty t\,C\,dt}{AUC} \quad (4)$$

$$VRT = \frac{\int_0^\infty (t - MRT)^2\,C\,dt}{AUC} \quad (5)$$

where C is the drug concentration at time t. The MRT and VRT have advantages over other methods of calculating drug bioavailability in that they reflect not only the extent of availability but also the rate of transit of drug molecules through the body. They are also independent of the pharmacokinetic model [159].

On the other hand, the determination of total drug absorption in intact animals is relatively complicated. Applicable information is usually gathered from the following sources.

1. Blood levels: A majority of the animal ADME studies are conducted during the preclinical phase of drug development when relevant metabolism data are either unavailable or fragmentary at best. Therefore, only the composite of all metabolites plus the parent drug can be monitored, that is, as total radioactivity. Although t_{max} and C_{max} of the radiotracer may be indicative of the rate of absorption, the complex nature of the AUC value for total radioactivity requires that this parameter be used with caution. If it is assumed that a drug that is completely absorbed into the portal circulation is eliminated both via renal excretion and by hepatic metabolism to form a single metabolite, the AUC of the parent drug after oral administration will be smaller but that of the metabolite will be greater than the respective values after intravenous dosing because of first-pass metabolism. The magnitude of the difference is dependent on the ratio of the renal versus hepatic clearance of the drug [160]. The decrease in AUC of the parent drug may or may not be equal to the increase in AUC of the metabolite, depending on the relative distribution volumes of the two species. Consequently, the oral-intravenous AUC ratio of total radioactivity (drug + metabolite) can be smaller than, equal to, or even greater than unity, regardless of the extent of absorption. In fact, this ratio would be a true representation of the fraction of dose absorbed only if the dose demonstrates little or no first-pass

effect and is studied in a dose range without saturating its usual elimination pathways.

2. Urinary excretion: The ratio of cumulative urinary recovery of radioactivity after oral and intravenous drug administration is a commonly accepted index of absorption [161163], provided that the excretion pattern (urine/feces ratio) of absorbed radioactivity does not vary significantly with dose and route of administration. This condition can be verified to some extent by showing similar patterns of urinary metabolites following oral and intravenous doses. Under other circumstances, the urinary recovery of an orally administered radioactive dose can still provide useful information, since it represents the minimum amount of drug absorbed [164].

3. Parent drug in feces: This method simply assumes that any intact drug recovered in the feces represents the unabsorbed portion of dose. It neglects the possibility of biliary excretion of unchanged drug, nor does it consider the secretion of certain drugs [165169] from blood directly into the gut lumen (exsorption). On the other hand, the amount of drug unabsorbed could be underestimated owing to the possibility of drug metabolism at the gut epithelium [170] or by intestinal microflora [171]. Therefore, unchanged drug in feces should not be used as the sole absorption index but may serve as a secondary, supportive indicator.

4. Excretion in bile duct-cannulated animals: If properly conducted to maintain physiological conditions, experiments using bile duct-cannulated animals can yield definitive information concerning the extent of drug absorption [172]. Following oral administration of a radiolabeled compound, the total recovery of radioactivity in urine and bile would indicate the minimum amount of drug absorbed; any radioactive material present in feces probably represents unabsorbed drug. In addition, the fraction of radioactivity excreted in the bile that undergoes enterohepatic circulation can be calculated after intravenous drug administration using the method of Tse et al. [173], as follows:

$$F_a = \frac{1 - AUC/AUC^*}{F_b} \quad (6)$$

where F_b is the fraction of drug in the body that is excreted in bile and F_a is the fraction of the excreted drug in bile subsequently reabsorbed from the gut. AUC* and AUC are the areas under blood level versus time curves after administering the same dose to control animals and to bile duct-cannulated animals in which biliary recycling is interrupted, respectively. Equation (6) has been used satisfactorily in determining the enterohepatic circulation of temazepam [173] and diflunisal [174] in rats. F_a can also be estimated without blood concentration data, provided the cumulative fecal and biliary excretion in control

and bile duct-cannulated animals, respectively, are measured [173]:

$$F_a = \frac{F_b - F_f}{F_b (1 - F_f)} \quad (7)$$

In Equation (7), F_f is the fraction of the administered radioactivity recovered in the feces of control animals.

A combination of two or more of these indices is often necessary to provide good insight into the absorption phenomenon. Although blood concentration analysis can reveal the onset and rate of drug absorption, the bile duct-cannulated animal model is usually more reliable for the assessment of the extent of absorption. A potential flaw associated with the latter, however, is the necessity of surgical intervention, which could induce stress in the animal. The oral/intravenous urine quotient in intact animals is a useful indicator of absorption for drugs that are predominantly excreted in urine. This method may not be ideal if the renal route represents only a minor pathway of drug excretion: any experimental error in urine collection or analysis would tend to result in relatively large deviations in absorption estimates [175].

b. Distribution The concentrations of drug-related material (total radioactivity) in major tissues and organs reveal the degree of transient exposure of specific organs to the drug. Comparison of tissue and circulating concentrations can give a direct indication of the relative affinity of the drug to tissue and blood components. With adequate sampling at appropriate intervals after a single dose, the half-life of drug elimination from each organ can be calculated. The results are useful in predicting the extent of drug accumulation in tissues and organs, which can be verified by multiple dosing experiments. Furthermore, selected tissue and biofluid concentrations obtained in placental transfer studies are used to determine the extent of fetal exposure to the drug taken during pregnancy.

An important use of tissue concentration and half-life data is for the projection of radioactivity exposure to human subjects during clinical ADME studies. The method of calculation is detailed in Chapter 13. This information is required by most institutional review boards before approving study protocols that involve the administration of radiolabeled drugs to humans.

c. Metabolism As indicated previously, the doses employed in nonclinical ADME studies usually encompass the relatively broad range used in the subchronic toxicity trial. This provides an excellent opportunity to examine the potential effect of dose size on drug metabolism [176178]. An increase in dose frequently leads to a change in the pattern of metabolites [179,180]. Furthermore, the influence of the route of administration on metabolite formation can also be investigated [181].

A variety of biological samples, that is, blood, urine, feces, bile, liver, and so on, are usually collected in ADME studies and can be used for metabolism

work. This not only facilitates the isolation of most of the metabolites formed but also provides information on the excretory pathways of these biotransformation products. Comparison of the metabolite patterns following a single dose and at steady state indicates if the drug induces [182184] or inhibits [185] its own metabolism.

The main objective of biotransformation studies in animals is to identify those species in which the metabolic fate of the drug is similar to that in humans. If this is not attainable, it may suffice to have two or more species which between them display all the metabolic pathways present in the human. The finding of similar metabolic pathways in animals and humans validates the use of certain animal species in lieu of humans in long-term safety studies. On the other hand, if a human metabolite is not formed to any appreciable extent in any toxicological species, that metabolite may need to be synthesized and itself tested for safety in an animal model.

d. Excretion One of the primary objectives of ADME studies is to show that the administered dose is readily excreted from the body, presumably after exerting its intended therapeutic effect. In order to facilitate the achievement of mass balance, radiolabeled drug is almost always used in these studies. By quantitative collection of excreta at sufficient frequency, it is possible not only to provide information on the overall excretion pattern, that is, percentage of dose in urine, feces, and so on, but also to allow an assessment of the rate of elimination.

More often, however, the elimination rate of a drug is evaluated in terms of its terminal half-life in blood. The half-life is a useful kinetic parameter that indicates the time required to attain and decay from steady-state conditions after instituting a particular dosing regimen. It can be determined from the slope of the terminal, log-linear phase of the blood concentration vs. time plot. The key to success with this method lies entirely in properly defining the terminal phase. As pointed out by Wagner [186], the true terminal phase is likely to be missed if blood samples are not collected for an adequate length of time or if a relatively insensitive assay method is used. These circumstances usually result in an underestimate of the elimination half-life. On the other hand, a problem almost unique to ADME studies using radiolabeled drugs has been attributed to the relatively high sensitivity of liquid scintillation counting. This has frequently led to a terminal phase of low but lasting blood concentrations, hence a relatively long half-life, often representing the half-life of the slowest eliminating metabolite fragment containing the radiolabel. The significance of this half-life on the accumulation of drug-related material upon chronic dosing is unfortunately difficult to determine. The simple method proposed by Benet [187], which judges the relevance of a half-life by its contribution to the total AUC, is not appropriate for evaluating total radioactivity data owing to the complex

nature of the concentration value. Nonetheless, it is possible that the long half-life represents only

a small fraction of the dose that is reversibly bound to and slowly released from a specific tissue depot. If such binding is saturable, the half-life will indeed not be predictive of accumulation. An interesting example of this phenomenon concerns the angiotensin-converting enzyme inhibitor enalaprilat. Enalaprilat is usually given as enalapril, the ethyl ester of enalaprilat that is well absorbed orally and rapidly and extensively hydrolyzed to the active diacid [188]. Repeated daily administration of enalapril to healthy volunteers resulted in little accumulation of enalaprilat [189], inconsistent with the reported terminal half-life of approximately 35 h following a single oral dose [190]. The investigators postulated that the terminal phase of the enalaprilat serum profile represents binding of enalaprilat to angiotensin-converting enzyme and that this binding involves a finite amount of drug, regardless of the dose administered. Thus, the terminal phase would effectively have a fixed or one-time contribution to accumulation, contrary to the additive contribution by successive doses in a linear process. It was suggested that under such circumstances, steady-state blood level or urinary recovery data be used to estimate an effective half-life, that is, one consistent with the observed accumulation of drug during chronic dosing. In ADME studies that include a multiple dosing regimen, the effective half-life ($t_{1/2,eff}$) can be calculated as follows [191,192]:

$$t_{1/2,eff} = \frac{0.693}{\omega} \quad (8)$$

where

$$\omega = -\frac{\ln (1 - 1/R)}{\tau} \quad (9)$$

In Equation (9), t is the dose interval and R is the accumulation ratio. The value of R is usually estimated by one of the following methods:

$$R = \frac{AUC_{0-\tau(ss)}}{AUC_{0-\tau(1)}} \quad (10)$$

$$R = \frac{C_{min(ss)}}{C_{min(1)}} \quad (11)$$

where $AUC_{0-t(1)}$ and $AUC_{0-t(ss)}$ are the AUC values during a dose interval following a single dose and at steady state, respectively, and $C_{min(1)}$ and $C_{min(ss)}$ are the blood concentrations immediately prior to the administration of the second dose and any dose at steady state, respectively [193]. Equation (10) is the preferred method if sufficient serial blood samples are available after dosing on both day 1 and at steady state to determine the respective AUC values. Equation (11) is more susceptible to error in that excessive weight is placed on a single

determinant $C_{min(1)}$, even though $C_{min(ss)}$ can usually be obtained as the mean of several predose concentrations at steady state.

Thus, the relevance of the half-life obtained from a single dose in predicting drug or radioactivity accumulation and the time required to reach steady state should be verified by multiple dosing experiments. It also should be noted that chronic administration of a drug can lead to altered elimination characteristics, such as induced metabolism, which may or may not be reflected in a change in its elimination half-life in blood. For example, increased first-pass metabolism without a significant effect on subsequent drug disappearance rate has been reported for drugs subject to a high hepatic (flow-limited) clearance [194].

The excretion data obtained from animals are useful in the design of human ADME studies. Despite the marked differences in the relative urine and bile flow rates among various species (Table 5), which could lead to differences in the relative importance of the renal and biliary excretory pathways, the total recovery of administered radioactivity should be similar among species. Thus, if the combined recovery in animal urine and feces is considerably less than the amount administered, it would be advisable to collect additional sample types, such as exhaled air if the drug is ^{14}C-labeled, in the subsequent human ADME study. The half-lives in preclinical species can provide guidance in the selection of an adequate duration of sample collection.

B. Other Registration Studies

1. Blood-Plasma Ratio and Protein Binding

Drugs are delivered to and from tissues in whole blood, yet the ability of a drug to enter extravascular spaces is largely determined by its distribution characteristics within the blood components. Drug in blood may be distributed into erythrocytes or plasma water or may be reversibly bound to plasma proteins. The extent to which a drug is protein-bound depends upon the affinity of the

Table 5 Average Bile and Urine Flow Rates (ml/kg/day) in Different Species

Species	Bile Flow	Urine Flow
Mouse	100	50
Rat	90	60
Rabbit	120	60
Dog	12	30
Rhesus monkey	25	75
Human	5	20

Source: Data from Reference 17.

protein for the drug, the number of binding sites on the protein molecule for that particular drug, and the concentrations of both drug and protein.

Protein-bound drugs are essentially confined to the plasma volume. In this state they may also be devoid of any pharmacological activity, as is the case for most antibiotics [195]. Thus, both the pharmacokinetic and pharmacodynamic properties of a drug can be influenced by plasma protein binding [196203]. Therefore, protein-binding studies usually are conducted early in the course of drug development in order to allow proper interpretation of data in subsequent pharmacokinetic studies. Knowledge of the blood-plasma distribution ratio may also be useful to the bioanalyst concerned with assay sensitivity in selecting an optimal sample type (blood or plasma) in future studies.

Blood-plasma distribution ratio and plasma protein binding are usually evaluated by in vitro studies. Fresh blood tested immediately after collection from animals [204] and humans [205] is preferred to preserved blood. The use of an indwelling venous catheter for blood collection [206] and the presence of heparin in the blood sample [207210] are additional factors that can affect the results of protein binding studies. The importance of radiochemical purity of the radiolabeled drug used for determining plasma protein binding must also be stressed [211213].

The fresh blood is spiked with radioactive drug and incubated at 37°C for about 30 min. Aliquots of blood are removed for liquid scintillation counting, and plasma is separated from the remaining blood by centrifugation. Aliquots of plasma are then pipetted for radioactivity determination. The fraction of drug in blood that is distributed to red blood cells (fBC) is given by the following equation [214]:

$$f_{BC} = 1 - (1 - H)\frac{C_P}{C_B} \quad (12)$$

where H is the hematocrit and CP and CB are drug concentrations in plasma and whole blood, respectively. The binding of drug in plasma can be determined by a number of techniques, including equilibrium dialysis, ultrafiltration, ultracentrifugation, and gel filtration [215]. Each of these methods has demonstrated certain advantages and disadvantages, but the first two appear to be most popular among routine users. After ultrafiltration or at dialysis equilibrium, the free fraction of drug in plasma is determined from the ratio of drug concentration in the ultrafiltrate or dialysate to that in the original plasma. With both methods, control experiments must be performed to correct for concentration-dependent nonspecific binding of drug to the apparatus [216]. Other sources of error to which equilibrium dialysis may be especially prone include difficulties in attaining equilibrium [217220] and volume shifts across the membrane due to osmotic

differences between the buffer and plasma or serum [212,221223]. A relatively new method for measuring plasma protein binding is microdialysis [224226].

The procedure involves perfusing a microdialysis probe placed in plasma with a perfusion media. Available data suggest that microdialysis introduces no volume change, has a low possibility of nonspecific binding compared to ultrafiltration and equilibrium dialysis, and does not disturb the equilibrium of binding [227]. It also provides potential use for in vivo determination of protein binding.

Drug-plasma protein binding is usually studied over a concentration range representative of the therapeutic drug levels in humans. The results would reveal any concentration dependency in the extent of binding. The protein responsible for the binding of most drugs is albumin, which forms about one-half of the total plasma proteins. Nonetheless, many basic drugs often bind to a1-acid glycoprotein. For drugs that are extensively (> 90%) bound, it is desirable to identify the principal drug-binding protein using specific protein solutions. The affinity of the protein for the drug as indicated by the association constant and the number of binding sites on each protein molecule can be deduced using mathematical models [228230], although these specific parameters tend to have little practical value in drug development.

Protein-binding studies are usually conducted in human plasma as well as plasma from various animal species since interspecies differences in drug binding are common [231]. If the degree of binding in human plasma is high and if the therapeutic index of the drug is known to be low, competitive binding studies should be performed using plasma spiked with therapeutic agents that are likely to be administered concomitantly with the test drug [232]. Any effect of disease states on the protein binding of this type of drug could also be of particular clinical significance [233,234] and therefore warrants further investigation.

2. Placental Transfer

Owing to legal and ethical restrictions, placental transfer studies in humans have been largely confined to the comparison of drug concentrations in umbilical vein and maternal blood at birth. An often nondefinitive characterization of the time course of placental drug transfer can then be deduced by plotting fetal-maternal concentration ratios from different subjects as a function of the dose-delivery interval [235237]. In contrast, animal studies are not usually limited in this regard, and the increased flexibility of experimental design allows for the collection of data that would permit the evaluation of a variety of pharmacokinetic models [238,239]. Studies using ovine maternal-fetal units have described the rate and extent of transplacental passage of drugs from ewe to fetus and vice versa, thus providing insight into important underlying mechanisms, such as the differential binding of drug to maternal and fetal plasma proteins, placental drug clearance, and fetal drug elimination [240244]. The major advantage of using sheep is the

relatively large size of the fetus, which permits the implantation of

catheters for fetal blood collection without sacrificing the animal. Similar studies in rats would necessitate sampling from sacrificed animals [245,246].

In drug development, the primary purpose of placental transfer studies is to examine the overall extent of fetal exposure to drug that is administered to the pregnant mother, perhaps with less emphasis on the rate of the transfer process [247]. It has been stated that for both reversibly acting drugs and drugs that produce irreversible effects in the fetus, the ratio of the steady-state drug concentration in the fetus to that in the mother during chronic dosing throughout pregnancy may be used as the index of relative drug exposure [239]. The studies are often conducted in rabbits, rats, or mice, the species commonly used for reproductive toxicity studies.

Female animals are mated with males of the same source and strain. A clear and concise description of the handling and mating of rats, mice, and rabbits can be found in a text by Taylor [248]. Some breeding characteristics of these species are summarized in Table 6 [249]. For rats and mice, mating is confirmed by the presence of vaginal plug, an approximately 3 × 5 mm coagulated mass of sperm, on the bottom of the cage or in the vagina. A vaginal smear is performed only if no plug is found. The day of mating is generally considered day 0 of gestation.

The test compound is usually administered once daily, beginning on day 6 postconception (PC) and continuing through days 15 or 18 PC depending on the species (Table 6). Dosing prior to day 6 PC may kill the embryo before it implants.

Groups of three or more animals are sacrificed during various stages of pregnancy, for example, at the same time after dosing on days 10 and 13 PC and after the final dose. Additional animals are sacrificed at designated times after the last dose in order to obtain an indication of the rate of drug removal from

Table 6 Some Breeding Characteristics of the Mouse, Rat, and Rabbit

Species	Ovulation Interval (Days)	Detection of Mating	Accepted Period of Dosing to Cover Organogenesis (Days Postconception)	Average Duration of Gestation (Days)
Mouse	45	Vaginal plug, or sperm in smear	615	19
Rat	45	Vaginal plug, or sperm in smear	615	22

Rabbit	Induced by mating	Copulation	618	31

Source: Data from Reference 249.

the body. From each sacrificed animal, maternal blood and major excretory organs, such as liver and kidneys, as well as amniotic fluid, placentas, ovaries, uterus, corpora lutea, and fetuses, are collected for analysis.

Extra animals should be mated and carried through the study to ensure the availability of a sufficient number of pregnant animals. This allows for misdoses or spontaneous abortions.

3. Drug Passage into Milk

With the upsurge in breastfeeding in recent years, there has been a reasonable concern about infant exposure to drugs via breast milk. The physiological principles of drug transfer to human breast milk have been reviewed [250], and compartmental models have been proposed to describe the kinetics of this process [250,251]. A primary determinant of relative drug concentrations in milk and plasma is the degree of drug ionization, although additional factors, such as lipid solubility and differential drug binding to plasma and milk proteins, are also important [252254]. Given that permeation of the mammary epithelium occurs primarily by passive diffusion [255], the ratio of unbound drug concentration in milk to that in plasma (M/P) for a lipid-soluble drug can be estimated by the following rearrangements of the Henderson-Hasselbach equation [256]:

$$\frac{M}{P} = \frac{1 + 10^{(pHm - pKa)}}{1 + 10^{(pHb - pKa)}} \quad \text{for a weak acid} \tag{13}$$

$$\frac{M}{P} = \frac{1 + 10^{(pKa - pHm)}}{1 + 10^{(pKa - pHb)}} \quad \text{for a weak base} \tag{14}$$

where pHm and pHb represent the pH of milk (approximately 7.0) and blood (7.4), respectively. In order to account for the varying fat and protein contents that may alter the amount of drug bound to milk components, the total milk to plasma ratio can be obtained by multiplying Equations (13) and (14) by the factor fup/[fum. (S/M)], where fup and fum are the fractions of drug unbound in plasma and milk, respectively, and S/M is the skimmed to whole milk drug concentration ratio [257]. Measuring these variables in vitro also may permit extrapolation of results obtained from animal models to humans, which is otherwise difficult. For example, rabbit milk contains 5 to 10 times as much fat and protein as human milk, which would imply greater accumulation of drugs that bind to proteins and milk fat [258].

For investigational new drugs, drug excretion in milk can be evaluated only in laboratory animals because of obvious legal and ethical concerns. Usually, results obtained from the rat or rabbit are used to project the potential risk to breast-fed infants. In a typical study in rats, 2030 animals in their first lactation are used. The litter size is adjusted to about 10

within 12 days following parturition. The test drug, usually radiolabeled to facilitate quantitative deter-

mination of the parent compound and metabolites, is administered to the dams 810 days postparturition. The rats are then organized into groups for milk and blood collection at designated times postdose. All sucklings are removed from the dams several hours before milking. Oxytocin, 1 IU per rat, is given intravenously 5 min before each collection of milk to stimulate milk ejection. The usual yield of milk from each rat is about 1 ml. Blood or plasma is obtained by exsanguination immediately after milking.

The extent of drug passage into milk can be estimated as the milk-plasma ratio of drug concentrations at each sampling time or that of the AUC values. This ratio is then applied to the average plasma concentration in humans to obtain the average milk level following a given dose. Based on an approximate milk consumption of 150 ml/kg per day by an average infant [259], the quantity of drug received in the milk can be readily calculated. For example, if data in the rat show a milk-plasma drug AUC ratio of 5, an average plasma concentration of 1 ng/ml observed after a 5 mg dose would correspond to an average milk level of 5 ng/ml. Therefore, the maximum amount of drug that an infant could be exposed to by ingesting 1 L of milk per day would be 5 μg, that is, about 0.1% of the adult dose. Indeed, available data in humans have shown that the quantity of drug administered via nursing is considerably less than 1% of the adult dose in most cases [260].

C. Toxicokinetic Studies

1. Definition

Toxicokinetics can be loosely defined as applied pharmacokinetics in nonclinical toxicity trials. As such, the subject normally concerns pharmacokinetic observations at elevated dose levels far exceeding that employed for therapeutic efficacy. Under these circumstances, a number of nonlinear pharmacokinetic processes including dose-limited absorption, saturated first-pass effect, altered drug distribution and protein binding, saturated metabolism, as well as drug-induced changes in body physiology may occur, leading to drug disposition characteristics different from those observed following relatively low doses. Therefore, it is important to monitor the drug levels in animals in toxicity studies in order to ascertain adequate drug exposure from all doses tested [261]. Interspecies comparison of drug toxicity based solely on dose (mg) per body weight or body surface area [262] is no longer acceptable in general.

2. Objectives

The specific objectives of toxicokinetics include:

1. Assessing the average exposure achieved and its relationship to dose level, time course

of the study, and gender of the animal tested.

2. Contribute to dose selection in nonclinical toxicity studies in order to produce adequate exposure. For example, it is normally necessary to continue dose escalation until the maximum tolerated dose (MTD) is identified. An exception would be if maximum exposure (plateau in a curvilinear AUC-dose plot) is reached at a lower dose level, in which case that dose could suffice as the top dose in the study.

3. Contribute to dose selection in initial clinical trials. The MTD found in a preclinical toxicity species should be qualified by the degree of exposure before it is used in determining the initial human dose.

4. Validate the choice of species and dose regimen in nonclinical toxicity studies. For example, although the MTD is often used as the top dose level in carcinogenicity studies, this concept may be replaced by consideration of exposure levels in the test species and in humans. It has been recommended that in carcinogenicity studies a dose that provides a systemic exposure in rodents that exceeds the maximum exposure in humans by 25-fold or greater should be acceptable as the highest dose [263].

5. Relate blood levels of test substance to the toxicological findings and contribute to the assessment of their relevance to clinical safety.

Proper selection of dose levels in chronic toxicity studies allows one to show a no effect level at one end of the spectrum and clear toxicity at the other. In carcinogenicity studies, it is likewise generally required that no toxicity be evident at the lowest dose whereas at the highest dose there should be toxicity without altering the normal life span other than as a result of the effects of tumors.

3. Commonly Measured Parameters

Due to its integration into safety testing, the focus of toxicokinetics is on addressing relevant toxicological concerns and not on characterizing the basic pharmacokinetic parameters of the drug. In general, the objectives of toxicokinetic analyses can be achieved by measuring the following three parameters: C_{max} (peak blood concentration), t_{max} (time to reach peak concentration following drug administration), and AUC (area under concentration-time curve), with modifications for special dosing conditions. For example, it would be more appropriate to determine the steady-state drug concentration in blood (C_{ss}) instead of C_{max} if the drug is administered by constant rate infusion.

4. Origin of Data.

If a suitable assay method is available, toxicokinetic analysis should be performed in virtually all phases and types of toxicologic testing [264]. These normally include:

1. Subchronic toxicity studies (4, 13, or 26 week; 2 week for dose-range finding), most frequently conducted in the rat, dog, and monkey

2. Chronic toxicity studies (52 week), usually conducted in the dog

3. Carcinogenicity (oncogenicity) studies (13 week for dose-range finding; 2 year for main study), usually conducted in the rat and mouse

4. Reproductive toxicity studies (primarily segment II), usually conducted in the rat and rabbit

A blood concentration-time profile for a dose interval, usually consisting of 58 time points during 24 h, is constructed following the initial dose and near the end of the study. From dogs and other large laboratory species, sufficient blood can be obtained in serial sampling to permit construction of a concentration-time profile for each animal. Nonetheless, it is desirable to minimize the number of blood samples in these studies in order to reduce the capacity and time required for sample analysis. Whenever possible, sampling times should be selected based on available information on the absorption and disposition kinetics of the drug from prior studies [265].

Serial sampling normally cannot be applied to actual animals on a rodent toxicity study because the trauma associated with frequent venipuncture and blood loss could cause adverse changes in physiology, thus jeopardizing the integrity of the toxicity trial. As a consequence, various strategies have been adopted by different laboratories in an attempt to circumvent this problem. Some investigators collect one sample from each rat, timed to capture approximately the peak or trough concentration. Others routinely carry satellite animals, which are groups of animals included in the design and conduct of the toxicity study and housed with the main-study animals, but used primarily for toxicokinetic sampling. Both of these approaches contain some intrinsic flaws. A single time point cannot be expected to represent uniformly the peak concentration since the time to peak can vary considerably between different dose levels. The results are therefore unsuitable for determining the dose-concentration relationship. Whereas the timing of the trough concentrations can be more easily controlled, the relatively low drug levels, especially after the first dose, often lead to concerns regarding assay sensitivity during an early phase of drug development. On the other hand, the use of satellite animals inevitably requires a greater number of animals, more drug substance, and increased labor for dosing and veterinary care. It also does not allow assessment of the individual exposure profiles of the animals used in the toxicologic evaluation. Therefore, recent efforts in rodent toxicokinetics have focused on new experimental designs and associated statistical procedures for making inferences about concentration-time behavior, without the need for satellite animals [266,267]. One such design is illustrated in Table 7.

In this example, there were six treatment groups determined by two genders and three

dose levels (low, mid, and high, excluding control) in a 4 week study. Each group consisted of 10 rats. Blood samples were collected following drug

Table 7 Design of a Rat Toxicokinetic Study Using Sparse Sampling Approach

		Day 1		Day 21	
Dose Group	Sampling Time (h Postdose)	Male No.	Female No.	Male No.	Female No.
Low	1	1, 2	11, 12	1, 2	11, 12
	2	3, 4	13, 14	3, 4	13, 14
	4	5, 6	15, 16	5, 6	15, 16
	8	7, 8	17, 18	7, 8	17, 18
	24	9, 10	19, 20	9, 10	19, 20
Mid	1	21, 22	31, 32	21, 22	31, 32
	2	23, 24	33, 34	23, 24	33, 34
	4	25, 26	35, 36	25, 26	35, 36
	8	27, 28	37, 38	27, 28	37, 38
	24	29, 30	39, 40	29, 30	39, 40
High	1	41, 42	51, 52	41, 42	51, 52
	2	43, 44	53, 54	43, 44	53, 54
	4	45, 46	55, 56	45, 46	55, 56
	8	47, 48	57, 58	47, 48	57, 58
	24	49, 50	59, 60	49, 50	59, 60

administration on days 1 and 21. Two different rats from each group were sampled at each of five times: 1, 2, 4, 8, and 24 h postdose. Each rat had blood drawn at the same time postdose on both days of blood sampling. The design of this method was influenced by the population approach in clinical pharmacokinetics [268,269], in which average pharmacokinetic behavior for a population is inferred from a few observations on many individuals. A technique for estimating the mean AUC from time zero to a finite time postdose when only one sample per subject is available, but with multiple subjects sampled at each of several time points postdose, has been described by Bailer [270]. The standard error of the estimated AUC can be determined and the equality of two mean AUC values can be tested statistically. Bailer's method does not depend on any models for pharmacokinetic response, nor on any assumptions about variance homogeneity. The tests and confidence intervals depend only on the assumption of normally distributed data. The method can be applied if two or more animals within each treatment group are sampled at each time point, as is the case in the present example. Furthermore, if the terminal elimination rate constant of the test compound is known from an independent study, the method can be extended to estimate the mean AUC from time zero to infinity [271].

In reproductive toxicity studies, maternal blood samples are usually collected at five to

eight selected times postdose on the first and last day of treatment. The concentration data will provide exposure monitoring and reveal any poten-

tial difference in pharmacokinetics between pregnant and nonpregnant animals. Opinions tend to vary, however, with respect to monitoring the exposure of the fetuses. At least in the United States, many laboratories do not routinely measure fetal drug concentrations, citing that it is difficult or impossible to extrapolate these data to humans. Others consider that adequate placental transfer (drug level in whole fetus ≥ 1% of that in maternal plasma) should be demonstrated for the validity of reproductive toxicity studies. No single guideline to this issue would be suitable for all test compounds. Perhaps fetal drug concentration analysis should only be required in the absence of fetal toxicity and if there is no overt maternal toxicity even at the highest dose used in the study.

A subject that warrants some consideration in toxicokinetics is the potential need to monitor the exposure to drug metabolites. Metabolite plasma levels are generally measured only if the metabolite appears to make a major contribution to the pharmacological response, either in addition to the parent drug or by itself when the parent drug is designed to act as a prodrug. Measurement of circulating metabolite levels also may not account for possible reactive intermediate metabolites, which could be an area of concern in assessing relevant exposure in carcinogenicity studies [272].

D. Miscellaneous Studies

1. Dosage Form Evaluation

The objective of screening new dosage forms and formulations in animal models is to weed out poorly performing candidates prior to bioavailability evaluation in humans. A rapid turnaround is necessary for this testing procedure to be truly useful to the formulator. Therefore, the study should be designed to require the analysis of a minimum number of samples and sample types. Blood concentrations are usually measured in three to six dogs using a crossover design for the determination of relative bioavailability [273].

2. In Vitro and In Situ Studies

The discovery and development of new drug entities can be facilitated by the use of appropriate in vitro model systems. A popular model for drug transport is the Caco-2 cell monolayer [274276]. The Caco-2 cell line, a human colon adenocarcinoma cell line, spontaneously differentiates into polarized, columnar cells representative of the small intestine [277]. These cells exhibit well-developed microvilli and a polarized distribution of brush border enzymes and, when grown on plastic surfaces or microporous membranes, form domes characteristic of normal, transporting epithelium [278,279]. The experimental procedures including cell culture and tests to determine cell morphology and monolayer formation, cell polarity, as well as transepithelial electrical resistance (TEER) have been described elsewhere [274,276]. Radiolabeled drug dissolved

in an appropriate medium is placed in the apical (luminal) side of a monolayer of Caco-2 cells grown on a Transwell polycarbonate membrane, and an equal volume of the corresponding medium without drug is added to the basolateral (serosal) chamber. The apical-to-basolateral transport of drug is evaluated by analyzing samples collected from the basolateral chamber at regular time intervals. If a data base can be built over time correlating the permeability of the monolayers with in vivo absorption, at least for a given drug class, in vitro experiments conducted during the early discovery phase could be a efficient means of providing valuable guidance in the selection of lead compounds for further development. Other advantages of in vitro models include the opportunity to elucidate the molecular mechanisms of drug transport across the intestinal epithelium, and the potential reduction in the use of animals.

Isolated intestinal segments of the rat [280282] or dog [283] have likewise been used to study the mechanism and rate of drug absorption in situ when such information cannot be readily derived from blood concentration data following oral administration in conscious animals. Similar surgical preparations have enabled the determination of site-specific absorption from different segments of the gastrointestinal tract [284]. This information is useful in the development of dosage forms, especially in the area of controlled-release drug delivery systems.

IV. Summary

Attempts have been made in this chapter to describe a basic program of nonclinical pharmacokinetic studies that contributes to drug development and appears to meet the usual requirements of new drug registration. When appropriate, pharmacokinetic principles have been discussed from a practical perspective, with emphasis on aiding decision-making and addressing specific regulatory concerns.

A current trend that will surely continue is the inclusion of pharmacokinetic analysis in nonclinical toxicity trials. This approach requires a great deal of collaboration between the toxicologist and the pharmacokineticist, from the planning of the study to the final reporting of results. As an integral part of a toxicity study, the pharmacokinetics work must be conducted in full compliance with current good laboratory practice (GLP) regulations [285] and audited by the institutional quality assurance unit.

Since an overwhelming majority of nonclinical pharmacokinetic studies are conducted in living animals, it is appropriate to close this chapter with a few words on the sensitive and often controversial issue of laboratory animal welfare. Even though science has made remarkable progress in recent decades, and viable alternatives to animal testing such as computer modeling and cell and tissue cultures are being used, we still cannot create an

organ, tissue, or cell. Therefore, animal studies are essential when complex biological systems must interact to

determine the effects of a compound. Presently they are required by all regulatory authorities before a new drug can be administered to humans. Given the continued reliance on animal testing, it is absolutely necessary that all scientists conducting these studies receive prior training in the humane treatment of animals. The 1986 amendments to the Animal Welfare Act strengthened standards for laboratory animal care and increased enforcement of the act [286]. Every institution in the United States that utilizes animals for research is now required to appoint an institutional animal care and use committee, whose primary responsibilities are to review all research protocols involving the use of animals, to ensure the proper care and treatment of laboratory animals in accordance with federal, state, and local regulations, and to conduct frequent inspections of all animal facilities. The use of animals in lieu of humans in preliminary and risky drug testing is a privilege, not a right. Only through a conscientious effort to avoid the misuse and unnecessary use of animals can we continue to enjoy this privilege.

References

1. Drug Information Association International Symposium, International Harmonization of Non-Clinical Drug Safety Requirements, Tokyo, Japan, October 2526, 1990.

2. First International Conference on Harmonization, Harmonization of Regulatory Requirements for Pharmaceutical Products, Brussels, Belgium, November 57, 1991.

3. T. Ishizaki and K. Tawara. Relationship between pharmacokinetics and pharmacodynamics of the beta adrenergic blocking drug sotalol in dogs. J. Pharmacol. Exp. Ther. 211:331337, 1979.

4. H. F. Schran, F. L. S. Tse, and S. I. Bhuta. Pharmacokinetics and pharmacodynamics of bromocriptine in the rat. Biopharm. Drug. Dispos. 6:301311, 1985.

5. L. B. Sheiner, D. R. Stanski, S. Vozeh, R. D. Miller, and J. Ham. Simultaneous modeling of pharmacokinetics and pharmacodynamics: Application to *d*-tubocurarine. Clin. Pharmacol. Ther. 25:358372, 1979.

6. N. H. G. Holford and L. B. Sheiner. Pharmacokinetic and pharmacodynamic modeling in vivo. Crit. Rev. Bioeng. 5:273322, 1981.

7. W. A. Colburn. Simultaneous pharmacokinetic and pharmacodynamic modeling. J. Pharmacokinet. Biopharm. 9:367388, 1981.

8. B. E. Dahlström, L. K. Paalzow, G. Segre, and Å. J. Agren. Relationship between morphine pharmacokinetics and analgesia. J. Pharmacokinet. Biopharm. 6:4153, 1978.

9. Z. Yu and F. L. S. Tse. Simultaneous modeling of pharmacokinetics and

pharmacodynamics using IMSL/IDL. Pharm. Res. 10:S382, 1993.

10. P. Francheteau, J.-L. Steimer, C. Dubray, and D. Lavene. Mathematical model for in vivo pharmacodynamics integrating fluctuation of the response: Application to

the prolactin suppressant effect of the dopaminomimetic drug DCN 203922. J. Pharmacokinet. Biopharm. 19:287309, 1991.

11. L. Aarons, J. W. Mandema, and M. Danhof. A population analysis of the pharmacokinetics and pharmacodynamics of midazolam in the rat. J. Pharmacokinet. Biopharm. 19:485496, 1991.

12. Physicians' Desk Reference, 48th ed. Medical Economics Data, Oradell, New Jersey, 1994, p. 1306.

13. H. A. Eder. The effect of diet on the transport of probucol in monkeys. Artery 10:105107, 1982.

14. K. J. Palin and C. G. Wilson. The effect of different oils on the absorption of probucol in the rat. J. Pharm. Pharmacol. 36:641643, 1984.

15. C. C. Peck, W. H. Barr, L. Z. Benet, J. Collins, R. E. Desjardins, D. E. Furst, J. G. Harter, G. Levy, T. Ludden, J. H. Rodman, L. Sanathanan, J. J. Schentag, V. P. Shah, L. B. Sheiner, J. P. Skelly, D. R. Stanski, R. J. Temple, C. T. Viswanathan, J. Weissinger, and A. Yacobi. Opportunities for integration of pharmacokinetics, pharmacodynamics, and toxicokinetics in rational drug development. Pharm. Res. 9:826833, 1992.

16. D. A. Smith. Integration of animal pharmacokinetic and pharmacodynamic data in drug safety assessment. Eur. J. Drug Metab. Pharmacokinet. 18:3139, 1993.

17. B. Clark and D. A. Smith. Pharmacokinetics and toxicity testing. Crit. Rev. Toxicol. 12:343385, 1984.

18. J. D. Veldhuis, J. F. Strauss, S. L. Silavin, and L. A. Kolp. The role of cholesterol esterification in ovarian steroidogenesis: Studies in cultured swine granulosa cells using a novel inhibitor of acyl coenzyme A: cholesterol acyltransferase. Endocrinology 116:2530, 1985.

19. F. L. S. Tse and J. M. Jaffe. Disposition of a silicon-containing amide, an inhibitor of acyl-CoA:cholesterol acyltransferase, in dog and rat. Biopharm. Drug Dispos. 8:437448, 1987.

20. K. J. Palin. Lipids and oral drug delivery. Pharm. Int. 6:272275, 1985.

21. F. L. S. Tse and J. M. Jaffe. Influence of high-fat meal on the absorption of a silicon-containing amide, an inhibitor of acyl-CoA:cholesterol acyltransferase, in man. Biopharm. Drug Dispos. 9:201210, 1988.

22. F. L. S. Tse and P. G. Welling. Prednisolone bioavailability in the dog. J. Pharm. Sci.

66:17511754, 1977.

23. M. E. Pickup. Clinical pharmacokinetics of prednisone and prednisolone. Clin. Pharmacokinet. 4:111128, 1979.

24. J. G. Gambertoglio, W. J. C. Amend, Jr., and L. Z. Benet. Pharmacokinetics and bioavailability of prednisone and prednisolone in healthy volunteers and patients: A review. J. Pharmacokinet. Biopharm. 8:152, 1980.

25. M. L. Rocci, Jr., N. F. Johnson, and W. J. Jusko. Serum protein binding of prednisolone in four species. J. Pharm. Sci. 69:977978, 1980.

26. B. B. Brodie. Of mice, microsomes and men. Pharmacologist 6:1226, 1964.

27. R. L. Dedrick. Animal scale-up. J. Pharmacokinet. Biopharm. 1:435461, 1973.

28. E. F. Adolph. Quantitative relations in the physiological constitutions of mammals. Science 109:579585, 1949.

29. R. L. Dedrick, K. B. Bischoff, and D. S. Zaharko. Interspecies correlation of plasma concentration history of methotrexate (NSC-740). Cancer Chemother. Rep. Part 1 54:95101, 1970.

30. H. Boxenbaum. Interspecies scaling, allometry, physiological time, and the ground plan of pharmacokinetics. J. Pharmacokinet. Biopharm. 10:201227, 1982.

31. J. Mordenti. Forecasting cephalosporin and monobactam antibiotic half-lives in humans from data collected in laboratory animals. Antimicrob. Agents Chemother. 27:887891, 1985.

32. H. Boxenbaum. Interspecies variation in liver weight, hepatic blood flow, and antipyrine intrinsic clearance: Extrapolation of data to benzodiazepines and phenytoin. J. Pharmacokinet. Biopharm. 8:165176, 1980.

33. E. A. Swabb and D. P. Bonner. Prediction of aztreonam pharmacokinetics in humans based on data from animals. J. Pharmacokinet. Biopharm. 11:215223, 1983.

34. Y. Sawada, M. Hanano, Y. Sugiyama, and T. Iga. Prediction of the disposition of b-lactam antibiotics in humans from pharmacokinetic parameters in animals. J. Pharmacokinet. Biopharm. 12:241261, 1984.

35. J. Mordenti. Man versus beast: Pharmacokinetic scaling in mammals. J. Pharm. Sci. 75:10281040, 1986.

36. P. J. McNamara. Interspecies scaling in pharmacokinetics. In Pharmaceutical Bioequivalence. Edited by P. G. Welling, F. L. S. Tse, and S. V. Dighe. Marcel Dekker, New York, 1991, pp. 267300.

37. R. L. Dedrick. Animal scale-up. In Pharmacology and Pharmacokinetics. Edited by T. Teorell, R. L. Dedrick, and P. G. Condliffe. Plenum Press, New York, 1974, pp. 117145.

38. L. E. Gerlowski and R. K. Jain. Physiologically based pharmacokinetic modeling: Principles and applications. J. Pharm. Sci. 72:11031127, 1983.

39. R. L. Dedrick, D. D. Forrester, J. N. Cannon, S. M. El Dareer, and L. B. Mellett. Pharmacokinetics of 1-b-d-arabinofuranosylcytosine (Ara-C) deamination in several species. Biochem. Pharmacol. 22:24052417, 1973.

40. R. J. Lutz, R. L. Dedrick, H. B. Matthews, T. E. Eling, and M. W. Anderson. A preliminary pharmacokinetic model for several chlorinated biphenyls in the rat. Drug Metab. Dispos. 5:386396, 1977.

41. K. B. Bischoff and R. L. Dedrick. Thiopental pharmacokinetics. J. Pharm. Sci.

57:13461351, 1968.

42. A. Bernareggi and M. Rowland. Physiologic modeling of cyclosporin kinetics in rat and man. J. Pharmacokinet. Biopharm. 19:2150, 1991.

43. R. Kawai. Role of blood cells uptake on cyclosporines pharmacokinetics. International Symposium on Blood Binding and Drug Transfer, Basel, September 1819, 1992.

44. F. G. King and R. L. Dedrick. Physiologic model for the pharmacokinetics of 2'-deoxycoformycin in normal and leukemic mice. J. Pharmacokinet. Biopharm. 9:519534, 1981.

45. F. F. Farris, F. G. King, R. L. Dedrick, and C. L. Litterst. Physiological model for the pharmacokinetics of cis-dichlorodiammineplatinum(II) (DDP) in the tumored rat. J. Pharmacokinet. Biopharm. 13:1340, 1985.

46. F. G. King, R. L. Dedrick, and F. F. Farris. Physiological pharmacokinetic modeling of cis-dichlorodiammineplatinum(II) (DDP) in several species. J. Pharmacokinet. Biopharm. 14:131155, 1986.

47. F. F. Farris, R. L. Dedrick, and F. G. King. Cisplatin pharmacokinetics: Applications of a physiological model. Toxicol. Letts. 43:117137, 1988.

48. F. G. King and R. L. Dedrick. Physiological pharmacokinetic parameters for cis-dichlorodiammineplatinum(II) (DDP) in the mouse. J. Pharmacokinet. Biopharm. 20:9599, 1992.

49. F. G. King, R. L. Dedrick, J. M. Collins, H. B. Matthews, and L. S. Birnbaum. Physiological model for the pharmacokinetics of 2,3,7,8-tetrachlorodibenzofuran in several species. Toxicol. Appl. Pharmacol. 67:390400, 1983.

50. K. Krishnan and M. E. Anderson. Physiological modeling and cancer risk assessment. In New Trends in Pharmacokinetics. Edited by A. Rescigno and A. K. Thakur. Plenum Press, New York, 1991, pp. 335354.

51. D. Krewski, J. R. Withey, L. F. Ku, and C. C. Travis. Physiologically based pharmacokinetic models: Applications in carcinogenic risk assessment. In New Trends in Pharmacokinetics. Edited by A. Rescigno and A. K. Thakur. Plenum Press, New York, 1991, pp. 355390.

52. L. J. Leeson, D. Adair, J. Clevenger, and N. Chiang. The in vitro development of extended-release solid oral dosage forms. J. Pharmacokinet. Biopharm. 13:493514, 1985.

53. R. L. Williams, R. A. Upton, L. Ball, R. L. Braun, E. T. Lin, W. Liang-Gee, and L. J. Leeson. Development of a new controlled-release formulation of chlorpheniramine maleate using in vitro/in vivo correlations. J. Pharm. Sci. 80:2225, 1991.

54. J. B. Dressman and K. Yamada. Animal models for oral drug absorption. In Pharmaceutical Bioequivalence. Edited by P. G. Welling, F. L. S. Tse, and S. V. Dighe. Marcel Dekker, New York, 1991, pp. 235266.

55. R. D. Smyth, K. A. Dandekar, F. H. Lee, A. F. DeLong, and A. Polk. Use of the dog in bioavailability and bioequivalence testing. In Animal Models for Oral Drug Delivery in Man: In Situ and In Vivo Approaches. Edited by W. Crouthamel and A. C. Sarapu. American Pharmaceutical Association, Washington, D. C., 1983, pp. 125148.

56. W. J. Hamilton. Textbook of Human Anatomy. Macmillan, London, 1957, p. 508.

57. A. C. Anderson. The Beagle As An Experimental Dog. Iowa State University Press,

Ames, Iowa, 1970, p. 226.

58. P. Bass. Physiological parameters of the GI tract that could affect absorption. Workshop on Oral Drug Delivery: Interface between Discovery and Development, National Institute of General Medical Sciences, Herndon, Virginia, December 57, 1993.

59. C. Y. Lui, G. L. Amidon, R. R. Berardi, D. Fleisher, C. Youngberg, and J. B. Dressman. Comparison of gastrointestinal pH in dogs and humans: Implications on the use of the beagle dog as a model for oral absorption in humans. J. Pharm. Sci. 75:271274, 1986.

60. H. Ogata, N. Aoyagi, N. Kaniwa, A. Ejima, T. Kitamura, T. Ohki, and K. Kitamura. Evaluation of beagle dogs as an animal model for bioavailability testing of cinnarizine capsules. Int. J. Pharm. 29:121126, 1986.

61. M. J. Swenson. Dukes Physiology of Domestic Animals. Cornell University Press, Ithaca, New York, 1970, p. 220.

62. J. B. Dressman. Comparison of canine and human gastrointestinal physiology. Pharm. Res. 3:123131, 1986.

63. F. N. Christensen, S. S. Davis, J. G. Hardy, M. J. Taylor, D. R. Whalley, and C. G. Wilson. The use of gamma scintigraphy to follow the gastrointestinal transit of pharmaceutical formulations. J. Pharm. Pharmacol. 37:9195, 1985.

64. H. Ogata, N. Aoyagi, N. Kaniwa, M. Koibuchi, T. Shibazaki, A. Ejima, T. Shimamoto, T. Yashiki, Y. Ogawa, Y. Uda, and Y. Nishida. Correlation of the bioavailability of diazepam from uncoated tablets in beagle dogs with its dissolution rate and bioavailability in humans. Int. J. Clin. Pharmacol. Ther. Toxicol. 20:576581, 1982.

65. N. Aoyagi, H. Ogata, N. Kaniwa, A. Ejima, H. Nakata, J. Tsutsumi, T. Fujita, and I. Amada. Bioavailability of indomethacin capsules in humans (III): Correlation with bioavailability in beagle dogs. Int. J. Clin. Pharmacol. Ther. Toxicol. 23:578584, 1985.

66. M. Bialer, M. Friedman, and J. Dubrovsky. Comparative pharmacokinetic analysis of a novel sustained release dosage form of valproic acid in dogs. Biopharm. Drug Dispos. 5:110, 1984.

67. M. Bialer, M. Friedman, and J. Dubrovsky. Pharmacokinetic evaluation of novel sustained-release dosage forms of valproic acid in humans. Biopharm. Drug Dispos. 6:401411, 1985.

68. M. Bialer, M. Friedman, and J. Dubrovsky. Relation between absorption half-life values of four novel sustained-release dosage forms of valproic acid in dogs and humans. Biopharm. Drug. Dispos. 7:495500, 1986.

69. W. Crouthamel and I. Bekersky. Preclinical evaluation of new drug candidates and drug delivery systems in the dog. In Animal Models for Oral Drug Delivery in Man: In Situ and In Vivo Approaches. Edited by W. Crouthamel and A. C. Sarapu. American Pharmaceutical Association, Washington, D.C., 1983, pp. 107123.

70. E. D. Purich and J. P. Hunt. The use of animal models in new drug evaluation. In Animal Models for Oral Drug Delivery in Man: In Situ and In Vivo Approaches. Edited by W. Crouthamel and A. C. Sarapu. American Pharmaceutical Association, Washington, D.C., 1983, pp. 163177.

71. R. Voges, B. R. Von Wartburg, and H. R. Loosli. Tritiated compounds for in vivo investigations: CAMP and 3H-NMR-spectroscopy for synthesis planning and process

control. In Synthesis and Applications of Isotopically Labeled Compounds 1985. Edited by R. R. Muccino. Elsevier, Amsterdam, 1986, pp. 371376.

72. C. R. Richmond, W. H. Langham, and T. T. Trujillo. Comparative metabolism of tritiated water by mammals. J. Cell. Comp. Physiol. 59:4553, 1962.

73. E. Azar and S. T. Shaw, Jr. Effective body water half-life and total body water in rhesus and cynomolgus monkeys. Can. J. Physiol. Pharmacol. 53:935939, 1975.

74. F. L. S. Tse, B. A. Orwig, T. Chang, L. Guarducci, and J. M. Jaffe. Effect of age on the disposition of a-[(dimethylamino)methyl]-2-(3-ethyl-5-methyl-4-isoxazolyl)-1H-3-indole-methanol in the rat. Drugs Exp. Clin. Res. 10:225234, 1984.

75. E. M. Boyd. Predictive drug toxicity: Assessment of drug safety before human use. Can. Med. Assoc. J. 98:278293, 1968.

76. F. L. S. Tse, J. M. Jaffe, A. E. Hassell, and H. F. Schran. Bioavailability of isradipine in young and old rats: Effect of mode of administration. J. Pharm. Pharmacol. 41:657660, 1989.

77. S. M. Singhvi, K. J. Kripalani, A. V. Dean, G. R. Keim, J. S. Kulesza, F. S. Meeker, Jr., J. J. Ross, Jr., J. M. Shaw, and B. H. Migdalof. Absorption and bioavailability of captopril in mice and rats after administration by gavage and in the diet. J. Pharm. Sci. 70:885888, 1981.

78. D. R. Van Harken and G. H. Hottendorf. Comparative absorption following the administration of a drug to rats by oral gavage and incorporation in the diet. Toxicol. Appl. Pharmacol. 43:407410, 1978.

79. B. L. Kamath, A. Yacobi, S. D. Gupta, H. Stampfli, M. Durrani, and C.-M. Lai. Bioavailability of *N*-acetylprocainamide from mixed diet in rats. Res. Commun. Chem. Pathol. Pharmacol. 32:299308, 1981.

80. R. D. Smyth, R. C. Gaver, K. A. Dandekar, D. R. Van Harken, and G. H. Hottendorf. Evaluation of the availability of drugs incorporated in rat laboratory diet. Toxicol. Appl. Pharmacol. 50:493499, 1979.

81. L. L. Augsburger. Powdered dosage forms. In Sprowl's American Pharmacy, 7th ed. Edited by L. W. Dittert. J. B. Lippincott, Philadelphia, 1974, p. 313.

82. F. L. S. Tse, T. Chang, B. Finkelstein, F. Ballard, and J. M. Jaffe. Influence of mode of intravenous administration and blood sample collection on rat pharmacokinetic data. J. Pharm. Sci. 73:15991602, 1984.

83. F. L. S. Tse, D. F. Nickerson, and R. Aun. Effect of isoflurane anesthesia on antipyrine pharmacokinetics in the rat. Pharm. Res. 9:15151517, 1992.

84. D. M. Cocchetto and T. D. Bjornsson. Methods for vascular access and collection of body fluids from the laboratory rat. J. Pharm. Sci. 72:465492, 1983.

85. W. M. Johannessen, I. M. Tyssebotn, and J. Aarbakke. Antipyrine and acetaminophen kinetics in the rat: Comparison of data based on blood samples from the cut tail and a cannulated femoral artery. J. Pharm. Sci. 71:13521356, 1982.

86. W. F. Bousquet, B. D. Rupe, and T. S. Miya. Endocrine and modification of drug responses in the rat. J. Pharmacol. Exp. Ther. 147:376379, 1965.

87. R. E. Stitzel and R. L. Furner. Stress-induced alterations in microsomal drug metabolism in the rat. Biochem. Pharmacol. 16:14891494, 1967.

88. F. L. S. Tse and P. G. Welling. Bioavailability of parenteral drugs. II. Parenteral doses other than intravenous and intramuscular routes. J. Parenter. Drug Assoc. 34:484495, 1980.

89. G. Lukas, S. D. Brindle, and P. Greengard. The route of absorption of intraperitoneally administered compounds. J. Pharmacol. Exp. Ther. 178:562566, 1971.

90. R. S. Pope. Small vessel cannulator. J. Appl. Physiol. 24:276, 1968.

91. B. Scharschmidt and P. D. Berk. A simple device to facilitate rapid blood sampling in small animals. Proc. Soc. Exp. Biol. Med. 143:364366, 1973.

92. R. A. Upton. Simple and reliable method for serial sampling of blood from rats. J. Pharm. Sci. 64:112114, 1975.

93. M. J. Jongen and A. W. Norman. A simplified cannulation procedure for pharmacokinetic experiments in rats. J. Pharmacol. Methods 17:271275, 1987.

94. P. N. Patsalos, M. S. Alavijeh, J. Semba, and Y. I. Lolin. A freely moving and behaving rat model for the chronic and simultaneous study of drug pharmaco-

kinetics (blood) and neuropharmacokinetics (cerebrospinal fluid): Hematological and biochemical characterization and kinetic evaluation using carbamazepine. J. Pharmacol. Toxicol. Methods 28:2128, 1992.

95. N. Terao and D. D. Shen. Alterations in serum protein binding and pharmacokinetics of 1-propranolol in the rat elicited by the presence of an indwelling venous catheter. J. Pharmacol. Exp. Ther. 227:369375, 1983.

96. B. Chindavijak, F. M. Belpaire, F. De Smet, and M. G. Bogaert. Alterations of the pharmacokinetics and metabolism of propranolol and antipyrine elicited by indwelling catheters in the rat. J. Pharmacol. Exp. Ther. 246:10751079, 1988.

97. F. Torres-Molina, J.-C. Aristorena, C. Garcia-Carbonell, L. Granero, J. Chesa-Jiménez, J. Pla-Delfina, and J.-E. Peris-Ribera. Influence of permanent cannulation of the jugular vein on pharmacokinetics of amoxycillin and antipyrine in the rat. Pharm. Res. 9:15871591, 1992.

98. H. B. Waynforth. Experimental and Surgical Technique in the Rat. Academic Press, London, 1980, p. 68.

99. S. M. Stearns and P. W. Lee. A rapid method for repeated collection of blood from the tail vein of rats. Lab. Anim. Sci. 34:395396, 1984.

100. R. P. Rand, A. C. Burton, and T. Ing. The tail of the rat, in temperature regulation and acclimatization. Can. J. Physiol. Pharmacol. 43:257267, 1965.

101. F. L. S. Tse, T. Chang, and J. M. Jaffe. Effect of butalbital and phenobarbital pretreatment on antipyrine clearance in the rat. Arch. Int. Pharmacodyn. Ther. 279:181194, 1986.

102. M. L. Horton, C. T. Olson, and D. W. Hobson. Femoral venipuncture for collection of multiple blood samples in the nonanesthetized rat. Am. J. Vet. Res. 47:17811782, 1986.

103. F. L. S. Tse and J. M. Jaffe. Preclinical Drug Disposition: A Laboratory Handbook. Marcel Dekker, New York, 1991, p. 55.

104. W. R. Hendee. Radioactive Isotopes in Biological Research. Wiley-Interscience, New York, 1973, p. 189.

105. S. Ullberg. Studies on the distribution and fate of S^{35}-labelled benzylpenicillin in the body. Acta Radiol. (Suppl.) 118:1110, 1954.

106. J. I. Williams, S. I. Bhuta, J. M. Jaffe, B. H. Migdalof, H. J. Schwarz, K. C. Talbot, J. F. Brouillard, P. Donatsch, C. Hodel, M. Lemaire, J. Meier, and A. Schweitzer.

Absorption, distribution, and excretion of fluproquazone in several animal species. Arzneimittelforschung 31(I):897904, 1981.

107. R. D. Irons and E. A. Gross. Standardization and calibration of whole-body autoradiography for routine semiquantitative analysis of the distribution of ^{14}C-labeled compounds in animal tissues. Toxicol. Appl. Pharmacol. 59:250256, 1981.

108. F. Keller and P. G. Waser. Quantification in macroscopic autoradiography with carbon-14. An evaluation of the method. Int. J. Appl. Radiat. Isot. 33:14271432, 1982.

109. J. R. Unnerstall, D. L. Niehoff, M. J. Kuhar, and J. M. Palacios. Quantitative receptor autoradiography using [^{3}H]ultrofilm: Application to multiple benzodiazepine receptors. J. Neurosci. Methods 6:5973, 1982.

110. A. Schweitzer, A. Fahr, and W. Niederberger. A simple method for the quantitation of ^{14}C-whole-body autoradiograms. Appl. Radiat. Isot. 38:329333, 1987.

111. P. W. Tomlinson, D. J. Jeffery, and C. W. Filer. A novel technique for assessment of biliary secretion and enterohepatic circulation in the unrestrained conscious rat. Xenobiotica 11:863870, 1981.

112. J. B. Furness and M. Costa. Adynamic ileus, its pathogenesis and treatment. Med. Biol. 52:8289, 1974.

113. T. R. Bates, M. Gibaldi, and J. L. Kanig. Solubilizing properties of bile salt solutions. I. Effect of temperature and bile salt concentration on solubilization of glutethimide, griseofulvin, and hexestrol. J. Pharm. Sci. 55:191199, 1966.

114. T. R. Bates, M. Gibaldi, and J. L. Kanig. Solubilizing properties of bile salt solutions. II. Effect of inorganic electrolyte, lipids, and a mixed bile salt system on solubilization of glutethimide, griseofulvin, and hexestrol. J. Pharm. Sci. 55:901906, 1966.

115. S. Feldman and M. Gibaldi. Physiologic surface-active agents and drug absorption. I. Effect of sodium taurodeoxycholate on salicylate transfer across the everted rat intestine. J. Pharm. Sci. 58:425428, 1969.

116. S. Feldman and M. Gibaldi. Physiologic surface-active agents and drug absorption. II. Comparison of the effect of sodium taurodeoxycholate and ethylenediaminetetraacetic acid on salicylamide and salicylate transfer across the everted rat small intestine. J. Pharm. Sci. 58:967970, 1969.

117. K. Kakemi, H. Sezaki, R. Konishi, T. Kimura, and M. Murakami. Effect of bile salts on the gastrointestinal absorption of drugs. I. Chem. Pharm. Bull. 18:275280, 1970.

118. K. Kakemi, H. Sezaki, R. Konishi, T. Kimura, and A. Okita. Effect of bile salts on the gastrointestinal absorption of drugs. II. Mechanism of the enhancement of the intestinal absorption of sulfaguanidine by bile salts. Chem. Pharm. Bull. 18:10341039, 1970.

119. S. Miyazaki, H. Inoue, T. Yamahira, and T. Nadai. Interaction of drugs with bile components. I. Effect of bile salts on the dissolution behavior of indomethacin and phenylbutazone. Chem. Pharm. Bull. 27:24682472, 1979.

120. A. Rahman, J. A. Barrowman, and A. Rahimtula. The influence of bile on the bioavailability of polynuclear aromatic hydrocarbons from the rat intestine. Can. J. Physiol. Pharmacol. 64:12141218, 1980.

121. S. Eriksson. Bile acids and steroids. Biliary excretion of the bile acids and cholesterol in bile fistula rats. Proc. Soc. Exp. Biol. Med. 94:578582, 1957.

122. F. L. S. Tse, F. Ballard, and J. M. Jaffe. Biliary excretion of [^{14}C]temazepam and its

metabolites in the rat. J. Pharm. Sci. 72:311312, 1983.

123. K. S. Pang and J. R. Gillette. A theoretical examination of the effects of gut wall metabolism, hepatic elimination, and enterohepatic recycling on estimates of bioavailability and of hepatic blood flow. J. Pharmacokinet. Biopharm. 6:355367, 1978.

124. H.-S. G. Chen and J. F. Gross. Pharmacokinetics of drugs subject to enterohepatic circulation. J. Pharm. Sci. 68:792794, 1979.

125. W. A. Colburn. Pharmacokinetic and biopharmaceutic parameters during enterohepatic circulation of drugs. J. Pharm. Sci. 71:131133, 1982.

126. P. Veng Pedersen and R. Miller. Pharmacokinetics and bioavailability of cimetidine in humans. J. Pharm. Sci. 69:394398, 1980.

127. T. A. Shepard, R. H. Reuning, and L. J. Aarons. Interpretation of area under the curve measurements for drugs subject to enterohepatic cycling. J. Pharm. Sci. 74:227228, 1985.

128. T. A. Shepard, R. H. Reuning, and L. J. Aarons. Estimation of area under the curve for drugs subject to enterohepatic cycling. J. Pharmacokinet. Biopharm. 13:589608, 1985.

129. P. Johnson and P. A. Rising. Techniques for assessment of biliary excretion and enterohepatic circulation in the rat. Xenobiotica 8:2736, 1978.

130. D. Greenslade, M. E. Havler, M. J. Humphrey, B. J. Jordan, and M. J. Rance. Species differences in the metabolism and excretion of fenclofenac. Xenobiotica 10:753760, 1980.

131. R. J. Parker, P. C. Hirom, and P. Millburn. Enterohepatic recycling of phenolphthalein, morphine, lysergic acid diethylamide (LSD) and diphenylacetic acid in the rat. Hydrolysis of glucuronic acid conjugates in the gut lumen. Xenobiotica 9:689703, 1980.

132. D. Brewster, M. J. Humphrey, and M. A. McLeavy. Biliary excretion, metabolism and enterohepatic circulation of buprenorphine. Xenobiotica 11:189196, 1981.

133. F. L. S. Tse, F. Ballard, and J. M. Jaffe. A practical method for monitoring drug excretion and enterohepatic circulation in the rat. J. Pharmacol. Methods 7:139144, 1982.

134. F. L. S. Tse, F. Ballard, J. M. Jaffe, and H. J. Schwarz. Enterohepatic circulation of radioactivity following an oral dose of [^{14}C]temazepam in the rat. J. Pharm. Pharmacol. 35:225228, 1983.

135. D. J. Paustenbach, G. P. Carlson, J. E. Christian, and G. S. Born. A comparative study of the pharmacokinetics of carbon tetrachloride in the rat following repeated inhalation exposures of 8 and 11.5 hr/day. Fundam. Appl. Toxicol. 6:484497, 1986.

136. S. Chakrabarti, R. Van Severen, and P. Braeckman. Influence of particle size and crystal shape on the dissolution rate of phenytoin. Farmacol. Tijdschr. Belg. 54:403411, 1977.

137. S. Chakrabarti, R. Van Severen, and P. Braeckman. Studies on the crystalline form of phenytoin. Pharmazie 33:338339, 1978.

138. F. Nimmerfall and J. Rosenthaler. Dependence of area under the curve on proquazone particle size and in vitro dissolution rate. J. Pharm. Sci. 69:605607, 1980.

139. H. A. Koeleman and M. C. B. Van Oudtshoorn. An evaluation of the biological availability of chloramphenicol. S. Afr. Med. J. 47:9499, 1973.

140. M. C. Meyer, G. W. A. Slywka, R. E. Dann, and P. L. Whyatt. Bioavailability of 14 nitrofurantoin products. J. Pharm. Sci. 63:16931698, 1974.

141. R. D. Schoenwald and P. Stewart. Effect of particle size on ophthalmic bioavailability of dexamethasone suspensions in rabbits. J. Pharm. Sci. 69:391394, 1980.

142. M. Dam, J. Christiansen, C. B. Kristensen, A. Helles, A. Jaegerskou, and M. Schmiegelow. Carbamazepine: A clinical biopharmaceutical study. Eur. J. Clin. Pharmacol. 20:5964, 1981.

143. G. T. McInnes, M. J. Asbury, L. E. Ramsay, J. R. Shelton, and I. R. Harrison. Effect of micronization on the bioavailability and pharmacologic activity of spironolactone. J. Clin. Pharmacol. 22:410417, 1982.

144. S. Chakrabarti, E. Moerman, and F. Belpaire. Bioavailability of phenytoin in dogs: Effect of crystal form and particle size. Pharmazie 34:242243, 1979.

145. A. D. J. Watson. Chloramphenicol in the dog: Observations of plasma levels following oral administration. Res. Vet. Sci. 16:147151, 1974.

146. F. L. S. Tse, J. M. Jaffe, K. A. Marty, and H. J. Schwarz. Effect of food, fluid and dosage form on the absorption of 52522, a potential antianxiety agent, in the dog. J. Pharm. Pharmacol. 36:5658, 1984.

147. P. G. Welling. Influence of food and diet on gastrointestinal drug absorption: A review. J. Pharmacokinet. Biopharm. 5:291334, 1977.

148. A. Melander. Influence of food on the bioavailability of drugs. Clin. Pharmacokinet. 3:337351, 1978.

149. R. D. Toothaker and P. G. Welling. The effect of food on drug bioavailability. Annu. Rev. Pharmacol. Toxicol. 20:173199, 1980.

150. P. G. Welling and F. L. S. Tse. The influence of food on the absorption of antimicrobial agents. J. Antimicrob. Chemother. 9:727, 1982.

151. P. G. Welling and F. L. S. Tse. Food interactions affecting the absorption of analgesic and anti-inflammatory agents. Drug-Nutr. Interact. 2:153168, 1983.

152. R. W. Marshall, O. M. Moreno, and D. A. Brodie. Chronic bile duct cannulation in the dog. J. Appl. Physiol. 19:11911192, 1964.

153. R. Jimenez, A. Esteller, and M. A. Lopez. Biliary secretion in conscious rabbits: Surgical technique. Lab. Animals 16:182185, 1982.

154. R. H. Dowling, E. Mack, J. Picott, J. Berger, and D. M. Small. Experimental model for the study of the enterohepatic circulation of bile in rhesus monkeys. J. Lab. Clin. Med. 72:169176, 1968.

155. J. Meszaros, F. Nimmerfall, J. Rosenthaler, and H. Weber. Permanent bile duct cannulation in the monkey. A model for studying intestinal absorption. Eur. J. Pharmacol. 32:233242, 1975.

156. R. C. Grimus and I. Schuster. The role of the lymphatic transport in the enteral

absorption of naftifine by the rat. Xenobiotica 14:287294, 1984.

157. W. N. A. Charman and V. J. Stella. Estimating the maximal potential for intestinal lymphatic transport of lipophilic drug molecules. Int. J. Pharm. 34:175178, 1986.

158. W. N. A. Charman and V. J. Stella. Transport of lipophilic molecules by the intestinal lymphatic system. Adv. Drug Deliv. Rev. 7:114, 1991.

159. K. Yamaoka, T. Nakagawa, and T. Uno. Statistical moments in pharmacokinetics. J. Pharmacokinet. Biopharm. 6:547558, 1978.

160. K. S. Pang. A review of metabolite kinetics. J. Pharmacokinet. Biopharm. 13:633662, 1985.

161. H. B. Hucker, S. C. Stauffer, A. J. Balletto, S. D. White, A. G. Zacchei, and B. H. Arison. Physiological disposition and metabolism of cyclobenzaprine in the rat, dog, rhesus monkey, and man. Drug Metab. Dispos. 6:659672, 1978.

162. J. E. Swagzdis, R. W. Wittendorf, R. M. DeMarinis, and B. A. Mico. Pharmacokinetics of dopamine-2 agonists in rats and dogs. J. Pharm. Sci. 75:925928, 1986.

163. H. B. Matthews, H. M. Chopade, R. W. Smith, and L. T. Burka. Disposition of 2,4-dinitroaniline in the male F-344 rat. Xenobiotica 16:110, 1986.

164. G. C. Bolton, G. D. Allen, C. W. Filer, and D. J. Jeffery. Absorption, metabolism and excretion studies on clauvlanic acid in the rat and dog. Xenobiotica 14:483490, 1984.

165. K. Arimori and M. Nakano. Transport of theophylline from blood to the intestinal lumen following i.v. administration to rats. J. Pharmacobiodyn. 8:324327, 1985.

166. K. Arimori and M. Nakano. Transport of phenobarbitone into the intestinal lumen and the biliary tract following i.v. administration to rats. J. Pharm. Pharmacol. 38:391393, 1986.

167. K. Arimori and M. Nakano. Transport of furosemide into the intestinal lumen and the lack of effect of gastrointestinal dialysis by charcoal in rats with acute renal failure. J. Pharmacobiodyn. 11:18, 1988.

168. H. Lennernäs and C.-G. Regårdh. Dose-dependent intestinal absorption and significant intestinal excretion (exsorption) of the b-blocker pafenolol in the rat. Pharm. Res. 10:727731, 1993.

169. A. B. Suttle, G. M. Pollack, and K. L. R. Brouwer. Intestinal secretion of ranitidine from in situ rat intestine. Pharm. Res. 10:S413, 1993.

170. W. A. Colburn, I. Bekersky, B. H. Min, B. J. Hodshon, and W. A. Garland. Contribution of gut contents, intestinal wall and liver to the first-pass metabolism of clonazepam in the rat. Res. Commun. Chem. Pathol. Pharmacol. 27:7390, 1980.

171. H. G. Boxenbaum, I. Bekersky, M. L. Jack, and S. A. Kaplan. Influence of gut microflora on bioavailability. Drug Metab. Rev. 9:259279, 1979.

172. H. P. A. Illing and J. M. Fromson. Species differences in the disposition and metabolism of 6,11-dihydro-11-oxodibenz[be]-oxepin-2-acetic acid (isoxepac) in rat, rabbit, dog, rhesus monkey, and man. Drug Metab. Dispos. 6:510517, 1978.

173. F. L. S. Tse, F. Ballard, and J. Skinn. Estimating the fraction reabsorbed in drugs undergoing enterohepatic circulation. J. Pharmacokinet. Biopharm. 10:455461, 1982.

174. J. H. Lin, K. C. Yeh, and D. E. Duggan. Effect of enterohepatic circulation on the pharmacokinetics of diflunisal in rats. Drug Metab. Dispos. 13:321326, 1985.

175. F. L. S. Tse, H. T. Smith, F. H. Ballard, and J. Nicoletti. Disposition of fluvastatin, an inhibitor of HMG-CoA reductase, in mouse, rat, dog, and monkey. Biopharm. Drug. Dispos. 11:519531, 1990.

176. P. A. J. Reilly, T. Inaba, D. Kadar, and L. Endrenyi. Enzyme induction following a single dose of amobarbital in dogs. J. Pharmacokinet. Biopharm. 6:305313, 1978.

177. P. G. Dayton and J. E. Sanders. Dose-dependent pharmacokinetics: Emphasis on phase I metabolism. Drug Metab. Rev. 14:347405, 1983.

178. G. Powis. Dose-dependent metabolism, therapeutic effect, and toxicity of anticancer drugs in man. Drug Metab. Rev. 14:11451163, 1983.

179. R. Mehta, P. C. Hirom, and P. Millburn. The influence of dose on the pattern of conjugation of phenol and 1-napthol in non-human primates. Xenobiotica 8:445452, 1978.

180. H. Koster, I. Halsema, E. Scholtens, M. Knippers, and G. J. Mulder. Dose-dependent shifts in the sulfation and glucuronidation of phenolic compounds in the rat in vivo and in isolated hepatocytes. The role of saturation of phenolsulfotransferases. Biochem. Pharmacol. 30:25692575, 1981.

181. J. C. Kapeghian, G. A. Burdock, and L. W. Masten. Effect of the route of administration on microsomal enzyme induction following repeated administration of methadone in the mouse. Biochem. Pharmacol. 28:30213025, 1979.

182. I. H. Patel, R. H. Levy, and W. F. Trager. Pharmacokinetics of carbamazepine-10,11-epoxide before and after autoinduction in rhesus monkeys. J. Pharmacol. Exp. Ther. 206:607613, 1978.

183. L. W. Masten, G. R. Peterson, A. Burkhalter, and E. L. Way. Tolerance to methadone lethality and microsomal enzyme induction in mice tolerant to and dependent on morphine. Drug Alcohol Depend. 5:2737, 1980.

184. L. W. Masten, S. R. Price, and C. J. Burnett. Microsomal enzyme induction following repeated oral administration of LAAM. Res. Commun. Chem. Pathol. Pharmacol. 20:119, 1978.

185. D. W. Schneck and J. F. Pritchard. The inhibitory effect of propranolol pretreatment on its own metabolism in the rat. J. Pharmacol. Exp. Ther. 218:575581, 1981.

186. J. G. Wagner. Pharmacokinetics and bioavailability. Triangle 14:101108, 1975.

187. L. Z. Benet. Pharmacokinetic parameters: Which are necessary to define a drug substance? Eur. J. Respir. Dis. (Suppl.) 65:4561, 1984.

188. E. H. Ulm. Enalapril maleate (MK-421), a potent, nonsulfhydryl angiotensin-converting enzyme inhibitor: Absorption, disposition, and metabolism in man. Drug Metab. Rev. 14:99110, 1983.

189. A. E. Till, H. J. Gomez, M. Hichens, J. A. Bolognese, W. R. McNabb, B. A. Brooks, F. Noormohamed, and A. F. Lant. Pharmacokinetics of repeated single oral doses of enalapril maleate (MK-421) in normal volunteers. Biopharm. Drug Dispos. 5:273280, 1984.

190. E. H. Ulm, M. Hichens, H. J. Gomez, A. E. Till, E. Hand, T. C. Vassil, J. Biollaz, H. R. Brunner, and J. L. Schelling. Enalapril maleate and a lysine analogue (MK-521): Disposition in man. Br. J. Clin. Pharmacol. 14:357362, 1982.

191. F. L. S. Tse and J. M. Jaffe. Pharmacokinetics of PN 200-110 (isradipine), a new

calcium antagonist, after oral administration in man. Eur. J. Clin. Pharmacol. 32:361365, 1987.

192. F. L. S. Tse, J. M. Jaffe, and A. Troendle. Pharmacokinetics of fluvastatin after single and multiple doses in normal volunteers. J. Clin. Pharmacol. 32:630638, 1992.

193. W. A. Colburn. Estimating the accumulation of drugs. J. Pharm. Sci. 72:833834, 1983.

194. G. Alvan, K. Piafsky, M. Lind, and C. von Bahr. Effect of pentobarbital on the disposition of alprenolol. Clin. Pharmacol. Ther. 22:316321, 1977.

195. G. N. Rolinson. The significance of protein binding of antibiotics in antibacterial chemotherapy. J. Antimicrob. Chemother. 6:311317, 1980.

196. J. J. Vallner. Binding of drugs by albumin and plasma protein. J. Pharm. Sci. 66:447465, 1977.

197. P. J. McNamara, J. T. Slattery, M. Gibaldi, and G. Levy. Accumulation kinetics of drugs with nonlinear plasma protein and tissue binding characteristics. J. Pharmacokinet. Biopharm. 7:397405, 1979.

198. S. Øie, T. W. Guentert, and T. N. Tozer. Effect of saturable binding on the pharmacokinetics of drugs: A simulation. J. Pharm. Pharmacol. 32:471477, 1980.

199. L. R. Peterson and D. N. Gerding. Influence of protein binding of antibiotics on serum pharmacokinetics and extravascular penetration: Clinically useful concepts. Rev. Infect. Dis. 2:340348, 1980.

200. E. M. Faed. Protein binding of drugs in plasma, interstitial fluid and tissues: Effect on pharmacokinetics. Eur. J. Clin. Pharmacol. 21:7781, 1981.

201. P. J. McNamara, M. Gibaldi, and K. Stoeckel. Volume of distribution terms for a drug (ceftriaxone) exhibiting concentration-dependent protein binding. I. Theoretical considerations. Eur. J. Clin. Pharmacol. 25:399405, 1983.

202. P. J. McNamara, M. Gibaldi, and K. Stoeckel. Volume of distribution terms for a drug (ceftriaxone) exhibiting concentration-dependent protein binding. II. Physiological significance. Eur. J. Clin. Pharmacol. 25:407412, 1983.

203. J. Huang and S. Øie. Hepatic elimination of drugs with concentration-dependent serum protein binding. J. Pharmacokinet. Biopharm. 12:6781, 1984.

204. R. C. Chou and G. Levy. Changes in plasma protein binding of drugs after blood collection from pregnant rats. J. Pharm. Sci. 71:471473, 1982.

205. A. J. Jackson, A. K. Miller, and P. K. Narang. Human blood preservation: Effect on in vitro protein binding. J. Pharm. Sci. 70:11681169, 1981.

206. N. Terao and D. D. Shen. Alterations in serum protein binding and pharmacokinetics of l-propranolol in the rat elicited by the presence of an indwelling venous catheter. J. Pharmacol. Exp. Ther. 227:369375, 1983.

207. M. Wood, D. G. Shand, and A. J. J. Wood. Altered drug binding due to the use of indwelling heparinized cannulas (heparin lock) for sampling. Clin. Pharmacol. Ther. 25:103107, 1979.

208. B. Silber, M. Lo, and S. Riegelman. The influence of heparin administration on the plasma protein binding and disposition of propranolol. Res. Commun. Chem. Pathol. Pharmacol. 27:419429, 1980.

209. C. A. Naranjo, J. G. Abel, E. M. Sellers, and H. G. Giles. Unaltered diazepam plasma

binding using indwelling heparinized cannulae for sampling. Br. J. Clin. Pharmacol. 9:103105, 1980.

210. C. A. Naranjo, E. M. Sellers, V. Khouw, P. Alexander, T. Fan, and J. Shaw. Variability in heparin effect on serum drug binding. Clin. Pharmacol. Ther. 28:545550, 1980.

211. T. D. Bjornsson, J. E. Brown, and C. Tschanz. Importance of radiochemical purity of radiolabeled drugs used for determining plasma protein binding of drugs. J. Pharm. Sci. 70:13721373, 1981.

212. F. D. Boudinot and W. J. Jusko. Fluid shifts and other factors affecting plasma protein binding of prednisolone by equilibrium dialysis. J. Pharm. Sci. 73:774780, 1984.

213. D. Mungall, Y. Y. Wong, R. L. Talbert, M. H. Crawford, J. Marshall, D. W. Hawkins, and T. M. Ludden. Plasma protein binding of warfarin: Methodological considerations. J. Pharm. Sci. 73:10001001, 1984.

214. M. Ehrnebo, S. Agurell, L. O. Boréus, E. Gordon, and U. Lönroth. Pentazocine binding to blood cells and plasma proteins. Clin. Pharmacol. Ther. 16:424429, 1974.

215. J. Steinhardt and J. A. Reynolds. Multiple Equilibria in Proteins. Academic Press, New York, 1969, pp. 4553.

216. C. J. Briggs, J. W. Hubbard, C. Savage, and D. Smith. Improved procedure for the determination of protein binding by conventional equilibrium dialysis. J. Pharm. Sci. 72:918921, 1983.

217. S. Øie and T. W. Guentert. Comparison of equilibrium times in dialysis experiments using spiked plasma or spiked buffer. J. Pharm. Sci. 71:127128, 1982.

218. P. J. McNamara and J. E. Bogardus. Effect of initial conditions and drug-protein binding on the time to equilibrium in dialysis systems. J. Pharm. Sci. 71:10661068, 1982.

219. S. S. Hwang and W. F. Bayne. Dynamic method for estimating the extent of plasma protein binding in a dialysis experiment. J. Pharm. Sci. 73:708710, 1984.

220. W. F. Bayne and S. S. Hwang. Effect of nonlinear protein binding on equilibrium times for different initial conditions. J. Pharm. Sci. 74:120123, 1985.

221. T. N. Tozer, J. G. Gambertoglio, D. E. Furst, D. S. Avery, and N. H. G. Holford. Volume shifts and protein binding estimates using equilibrium dialysis: Application to prednisolone binding in humans. J. Pharm. Sci. 72:14421446, 1983.

222. J. Huang. Errors in estimating the unbound fraction of drugs due to the volume shift in equilibrium dialysis. J. Pharm. Sci. 72:13681369, 1983.

223. J. J. Lima, J. J. MacKichan, N. Libertin, and J. Sabino. Influence of volume shifts on drug binding during equilibrium dialysis: Correction and attenuation. J. Pharmacokinet. Biopharm. 11:483498, 1983.

224. A. M. Herrera, D. O. Scott, and C. E. Lunte. Microdialysis sampling for determination of plasma protein binding of drugs. Pharm. Res. 7:10771081, 1990.

225. M. Ekblom, M. Hammarlund-Udenaes, T. Lundqvist, and P. Sjöberg. Potential use of microdialysis in pharmacokinetics: A protein binding study. Pharm. Res. 9:155158, 1992.

226. W. F. Elmquist, H. Yang, and B. Perry. The use of in vitro microdialysis to determine the plasma protein binding of cyclosporin A. Pharm. Res. 9:S337, 1992.

227. V. R. Shanbhag and M. M. Glynn. Comparison of equilibrium dialysis, ultrafiltration, and microdialysis for measurement of plasma protein binding. Pharm. Res. 10:S362,

1993.

228. G. Scatchard. The attractions of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. 51:660672, 1949.

229. J. E. Fletcher and A. A. Spector. Alternative models for the analysis of drug-protein binding. Mol. Pharmacol. 13:387399, 1977.

230. J. Romer and M. H. Bickel. A method to estimate binding constants at variable protein concentrations. J. Pharm. Pharmacol. 31:711, 1979.

231. H. Kurz and G. Friemel. Artspezifische Unterschiede der Bindung an Plasmaproteine. Naunyn Schmiedebergs Arch. Pharmacol. 257:3536, 1967.

232. F. L. S. Tse, D. F. Nickerson, and W. S. Yardley. Binding of fluvastatin to blood cells and plasma proteins. J. Pharm. Sci. 82:942947, 1993.

233. J. P. Tillement, F. Lhoste, and J. F. Giudicelli. Diseases and drug protein binding. Clin. Pharmacokinet. 3:144154, 1978.

234. K. M. Piafsky. Disease-induced changes in the plasma binding of basic drugs. Clin. Pharmacokinet. 5:246262, 1980.

235. G. Tomson, N.-O. Lunell, A. Sundwall, and A. Rane. Placental passage of oxazepam and its metabolism in mother and newborn. Clin. Pharmacol. Ther. 25:7481, 1979.

236. L. Padeletti, M. C. Porciani, and G. Scimone. Placental transfer of digoxin (betamethyldigoxin) in man. Int. J. Clin. Pharmacol. Biopharm. 17:8283, 1979.

237. O. M. Bakke and K. Haram. Time-course of transplacental passage of diazepam: Influence of injection-delivery interval on neonatal drug concentrations. Clin. Pharmacokinet. 7:353362, 1982.

238. G. Levy and W. L. Hayton. Pharmacokinetic aspects of placental drug transfer. In Fetal Pharmacology. Edited by L. O. Boréus. Raven Press, New York, 1973, pp. 2939.

239. H. H. Szeto. Pharmacokinetics in the ovine maternal-fetal unit. Annu. Rev. Pharmacol. Toxicol. 22:221243, 1982.

240. H. H. Szeto, L. I. Mann, A. Bhakthavathsalan, M. Liu, and C. E. Inturrisi. Meperidine pharmacokinetics in the maternal-fetal unit. J. Pharmacol. Exp. Ther. 206:448459, 1978.

241. H. H. Szeto, R. F. Kaiko, J. F. Clapp, R. W. Larrow, L. I. Mann, and C. E. Inturrisi. Urinary excretion of meperidine by the fetal lamb. J. Pharmacol. Exp. Ther. 209:244248, 1979.

242. H. H. Szeto, J. F. Clapp, III, R. W. Larrow, C. E. Inturrisi, and L. I. Mann. Renal tubular secretion of meperidine by the fetal lamb. J. Pharmacol. Exp. Ther. 213:346349, 1980.

243. H. H. Szeto, J. F. Clapp, III, R. W. Larrow, J. Hewitt, C. E. Inturrisi, and L. I. Mann. Disposition of methadone in the ovine maternal-fetal unit. Life Sci. 28:21112117, 1981.

244. G. W. Mihaly, D. J. Morgan, A. W. Marshall, R. A. Smallwood, S. Cockbain, D. MacLellan, and K. J. Hardy. Placental transfer of ranitidine during steady-state infusions of maternal and fetal sheep. J. Pharm. Sci. 71:10081010, 1982.

245. T. Nanbo. Pharmacokinetics in maternal-fetal unit after intravenous administration of p-phenyl benzoic acid to rat. J. Pharm. Dyn. 5:213221, 1982.

246. T. Nanbo. Change in pharmacokinetic character of p-phenyl benzoic acid in

developing fetus of rat. J. Pharm. Dyn. 5:222228, 1982.

247. F. L. S. Tse and D. Labbadia. Absorption and disposition of fluvastatin, an inhibitor of HMG-CoA reductase, in the rabbit. Biopharm. Drug Dispos. 13:285294, 1992.

248. P. Taylor. Practical Teratology. Academic Press, London, 1986, pp. 39.

249. A. K. Palmer. The design of subprimate animal studies. In Handbook of Teratology. Edited by J. G. Wilson and F. C. Fraser. Plenum Press, New York, 1978, p. 224.

250. J. T. Wilson, R. D. Brown, D. R. Cherek, J. W. Dailey, B. Hilman, P. C. Jobe, B. R. Manno, J. E. Manno, H. M. Redetzki, and J. J. Stewart. Drug excretion in

human breast milk: Principles, pharmacokinetics and projected consequences. Clin. Pharmcokinet. 5:166, 1980.

251. G. P. Stec, P. Greenberger, T. I. Ruo, T. Henthorn, Y. Morita, A. J. Atkinson, Jr., and R. Patterson. Kinetics of theophylline transfer to breast milk. Clin. Pharmacol. Ther. 28:404408, 1980.

252. H. C. Atkinson and E. J. Begg. The binding of drugs to major human milk whey proteins. Br. J. Clin. Pharmacol. 26:107109, 1988.

253. P. E. Macheras, M. A. Koupparis, and S. G. Antimisiaris. Drug binding and solubility in milk. Pharm. Res. 7:537541, 1990.

254. T. Stebler and T. W. Guentert. Bindings of drugs in milk: The role of casein in milk protein binding. Pharm. Res. 7:633637, 1990.

255. F. Rasmussen. Excretion of drugs by milk. In Concepts in Biochemical Pharmacology, Part 1. Edited by B. B. Brodie and J. R. Gillette. Springer-Verlag, Berlin, 1971, pp. 390402.

256. A. Goldstein, L. Aronow, and S. M. Kalman. Principles of Drug Action: The Basis of Pharmacology, 2nd ed. John Wiley and Sons, New York, 1974, pp. 145146.

257. J. C. Fleishaker, N. Desai, and P. J. McNamara. Factors affecting the milk-to-plasma drug concentration ratio in lactating women: Physical interactions with protein and fat. J. Pharm. Sci. 76:189193, 1987.

258. P. J. McNamara, D. Burgio, and S. D. Yoo. Pharmacokinetics of acetaminophen, antipyrine, and salicylic acid in the lactating and nursing rabbit, with model predictions of milk to serum concentration ratios and neonatal dose. Toxicol. Appl. Pharmacol. 109:149160, 1991.

259. P. N. Bennett. Drugs in breast milkan assessment of risk to the infant. III World Conference on Clinical Pharmacology and Therapeutics, Stockholm, Sweden, July 27August 1, 1986.

260. H. Vorherr. Drug excretion in breast milk. Postgrad. Med. 56:97104, 1974.

261. L. F. Chasseaud. The importance of pharmacokinetic/toxicokinetic and metabolic information in carcinogenicity study design. Drug Inf. J. 26:445455, 1992.

262. E. J. Freireich, E. A. Gehan, D. P. Rall, L. H. Schmidt, and H. E. Skipper. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. Cancer Chemother. Rep. 50:219244, 1966.

263. International Conference on Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use. Draft Consensus Text on Carcinogenicity: Guidance for dose selection for carcinogenicity studies of therapeutics, October 27, 1993.

264. D. A. Smith, M. J. Humphrey, and C. Charuel. Design of toxicokinetic studies. Xenobiotica 20:11871199, 1990.

265. W. M. Sallas. Sparse optimal sample times for estimating the area under the concentration versus time curve in toxicokinetic studies with large animals. Pharm. Res. 10:S350, 1993.

266. J. van Bree, J. Nedelman, F. Tse, J.-L. Steimer, W. Robinson, and W. Niederberger. Application of sparse sampling and non-linear mixed effects modelling in toxicokinetics. Pharm. Res. 10:S400, 1993.

267. J. R. Nedelman, E. Gibiansky, F. L. S. Tse, and C. Babiuk. Assessing drug exposure in rodent toxicity studies without satellite animals. J. Pharmacokinet. Biopharm. 21:323334, 1993.

268. L. B. Sheiner, B. Rosenberg, and K. L. Melmon. Modelling of individual pharmacokinetics for computer-aided drug dosage. Comp. Biomed. Res. 5:441459, 1972.

269. L. B. Sheiner, B. Rosenberg, and V. V. Marathe. Estimation of population characteristics of pharmacokinetic parameters from routine clinical data. J. Pharmacokinet. Biopharm. 5:445479, 1977.

270. A. J. Bailer. Testing for the equality of area under the curves when using destructive measurement techniques. J. Pharmacokinet. Biopharm. 16:303309, 1988.

271. J. Yuan. Estimation of variance for AUC in animal studies. J. Pharm. Sci. 82:761763, 1993.

272. A. Monro. What is an appropriate measure of exposure when testing drugs for carcinogenicity in rodents? Toxicol. Appl. Pharmacol. 112:171181, 1992.

273. H. Nosaka, K. Takagi, T. Hasegawa, Y. Ogura, Y. Mizukami, and T. Satake. Pharmacokinetics of theophylline in beagle dogs and asthmatic patients after multiple oral doses of sustained-release theophylline tablet formulation. Int. J. Clin. Pharmacol. Ther. Toxicol. 24:528535, 1986.

274. I. J. Hidalgo, T. J. Raub, and R. T. Borchardt. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 96:736749, 1989.

275. J. N. Cogburn, M. G. Donovan, and C. S. Schasteen. A model of human small intestinal absorptive cells. 1. Transport barrier. Pharm. Res. 8:210216, 1991.

276. I. J. Hidalgo, K. M. Hillgren, G. M. Grass, and R. T. Borchardt. Characterization of the unstirred water layer in Caco-2 cell monolayers using a novel diffusion apparatus. Pharm. Res. 8:222227, 1991.

277. A. R. Hilgers, R. A. Conradi, and P. S. Burton. Caco-2 cell monolayers as a model for drug transport across the intestinal mucosa. Pharm. Res. 7:902910, 1990.

278. E. Grasset, J. Bernabeu, and M. Pinto. Epithelial properties of human colonic carcinoma cell line Caco-2: effect of secretagogues. Am. J. Physiol. 248:C410C418, 1985.

279. I. Mohrmann, M. Mohrmann, J. Biber, and H. Murer. Sodium-dependent transport of P_i by an established intestinal epithelial cell line (CaCo-2). Am. J. Physiol.

250:G323G330, 1986.

280. L. S. Schanker, D. J. Tocco, D. B. Brodie, and C. A. M. Hogben. Absorption of drugs from the rat small intestine. J. Pharmacol. Exp. Ther. 123:8188, 1958.

281. J. T. Doluisio, N. F. Billups, L. W. Dittert, E. T. Sugita, and J. V. Swintosky. Drug absorption. I. An in situ rat gut technique yielding realistic absorption rates. J. Pharm. Sci. 58:11961200, 1969.

282. N. Schurgers, J. Bijdendijk, J. J. Tukker, and D. J. A. Crommelin. Comparison of four experimental techniques for studying drug absorption kinetics in the anesthetized rat in situ. J. Pharm. Sci. 75:117119, 1986.

283. D. C. Taylor, R. Grundy, and B. Loveday. Chronic dog intestinal loop model for studying drug absorption as exemplified by b-adrenoreceptor blocking agents, atenolol and propranolol. J. Pharm. Sci. 70:516521, 1981.

284. V. S. Patel and W. G. Kramer. Allopurinol absorption from different sites of the rat gastrointestinal tract. J. Pharm. Sci. 75:275277, 1986.

285. U. S. Food and Drug Administration, Good Laboratory Practice for Nonclinical Laboratory Studies, 21 CFR Part 58, 1987.

286. U. S. Congress, Office of Technology Assessment, Alternatives to Animal Use in Research, Testing, and Education, U.S. Government Printing Office, OTA-BA-273, Washington, D.C., 1986, p. 280.

13
Pharmacokinetics in Drug Discovery and Development: Clinical Studies

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I. Introduction

Prior to its first administration in humans, a new drug's pharmacology and safety will have been extensively tested in studies using in vitro and animal systems. These studies, consolidated in a Notice of Claimed Investigational Exemption for a New Drug (IND) and presented to the U.S. Food and Drug Administration (FDA), provide the basis of approval for limited, well-controlled trials of the new chemical entity (NCE) or biological product in humans [1]. A further prerequisite for the initiation of a specific clinical trial with the new drug is approval by the local investigational review board [2]. Other countries share the principle of regulatory and review board oversight of first time and subsequent use of a new drug in humans. Although procedures, review authority, and expectations for specific studies and data may differ between countries, considerable progress has been made in recent years to harmonize the new drug approval process [3].

Extrapolation from the preclinical to the clinical stage of new drug development involves considerable uncertainty and possible risk, despite the extensive pharmacological and toxicological tests in animals as mandated by the U.S. FDA, Japanese Ministry of Health and Welfare [4], and the European Community's (EC) [5] or other countries' regulatory bodies. Therefore, the progression from early trials with an NCE or biological product in a small number of patients or normal volunteers, to the extended clinical investigations during the later stages of drug development, is necessarily a methodical, and consecutive process involving several decision points. The Investigational New Drug Development Process as outlined in FDA form 1571 [6] divides the progression from an NCE's first time in humans to its approval for general use as a prescription drug into three distinct phases: phase I, consisting of safety studies, usually performed in less than 100 patients or normal volunteers; phase II, which evaluates the effectiveness and common short-term side effects in usually not more than several hundred patients; and phase III, which includes expanded controlled and uncontrolled clinical trials in several hundred to several thousand patients to gather pivotal information on safety and effectiveness. Clinical pharmacokinetic (PK) and bioavailability studies are an integral part of this consecutive process and represent a continuation of the pharmacokinetic and toxicokinetic investigations in animals performed prior to the IND submission. In the early stages of clinical development, PK studies provide an assessment of in-

trinsic drug properties, such as rates of absorption and elimination, or the degree of exposure to and accumulation of drug and/or its biotransformation products in human subjects, as well as an evaluation of the performance of dosage forms used and dosing schedule. In the latter stages of clinical development such studies could explain safety or efficacy issues, provide labeling information, and assess the performance of dosage forms other than those used in the clinical trials.

The 1977 FDA bioavailability and bioequivalence regulations and subsequent interpretations of these regulations elaborated the scope and formalized the conduct of several types of pharmacokinetic studies [8]. Since the first edition of this volume in 1988 [9] there has been further progress toward a broad regulatory, industrial, and academic consensus concerning pharmacokinetic activities in drug development [10,11]. Underlying this consensus is the realization of a need for flexibility, precluding an officially mandated checklist for the pharmacokinetic studies required as part of a new drug application (NDA, i.e., the regulatory basis for marketing a prescription drug substance). Thus, an approvable biopharmaceutics section of the NDA normally consists of a core group of clinical pharmacokinetics studies applicable to most new drugs, as well as supplemental studies related to the particular drug class or use. Considerable latitude exists in the number, design, and protocol of these studies, within the limits of ethical norms and accepted scientific principles and methodologies, as determined by peer review and institutional review board approval [2], and as governed by good clinical [12] and good laboratory practice [13]. Table 1 outlines the format of a typical NDA and one of its component chapters, i.e. Chapter VI, which represents the biopharmaceutics submission [14,15].

Table 2 summarizes typical core and supplemental pharmacokinetic studies contained in the biopharmaceutics section (Chapter VI) of an NDA. The type, protocol, and conduct of the core PK studies are well established. The scientific debate surrounding such specific issues as choice of statistical methodology for bioequivalence assessment [1620] has given way to a consensus of using confidence band [21] and other rigorous statistical bioavailability evaluations, discussed later in this chapter. There also has been considerable movement to enhance the core PK program by conducting concentration-controlled clinical trials, exploring pharmacokinetic-pharmacodynamic (PK-PD) correlations using such techniques as physiologically based PK-PD modeling, and performing population pharmacokinetics investigations. Activity in the population pharmacokinetics area has in the main focused on elaborating the mathematical-statistical approaches [2224]. There are as yet few examples demonstrating an advantage for better PK understanding of a new drug when studied by mixed effect modeling of sparse data sets in large numbers of subjects versus specific supplemental studies involving fewer

though extensively evaluated subject populations by classic methods [2527].

Table 1 Format for Human Pharmacokinetics and Bioavailability Section (Biopharmaceutics Submission) of an NDA Application

NDA Format		Biopharmaceutics Section (NDA Chapter VI) Format
I.	Index	A. Table of contents and summary of studies
II.	Summary	B. Summary of bioavailability and pharmacokinetic data and overall conclusions
III.	Chemistry, manufacturing, and controls	C. Summary of all formulations used in pivotal clinical trials and pharmacokinetic studies
IV.	Samples and labeling	D. Summary of analytical methodology employed
V.	Nonclinical pharmacology and toxicology	E. Summary description, specifications, and results of in vitro dissolution testing
VI.	Human pharmacokinetics and bioavailability	F. Individual study reports
VII.	Microbiology	Pilot or background studies Bioavailability and bioequivalence studies Pharmacokinetic studies In vitro dissolution studies
VIII.	Clinical data (part C contains clinical pharmacology studies)	Other in vivo studies using pharmacological or clinical end points. (Study reports with major clinical focus may be contained in Chapter VIII C as appropriate but should also be discussed and crossreferenced here.)

Table 2 serves to illustrate, not to prescribe, the various core and supplemental studies that may be included in biopharmaceutics submission. Such a submission should be designed within the context of the overall clinical and biopharmaceutical development program for, and take into consideration the specific characteristics of, the new drug entity in question. The conduct of some of the studies contained in Table 2 may be obviated by the results of population kinetic, PK/PD, or other such trials. As an alternative, such investigations could identify areas in which special supplemental studies would be desirable.

This chapter is organized into three general areas, discussing the pharmacokinetic studies typically performed during the clinical development phases I, II, and III, respectively. The

term phase I studies has been used interchangeably with normal volunteer studies by some investigators [28]. Because normal volunteer studies are conducted throughout the clinical development phases I through III, and may also be conducted to explore new dosage forms or for other purposes after an NDA has been filed or approved, this chapter reserves

Table 2 Pharmacokinetic Studies: Phase IIII

Study	Clinical Phase	Objective	Suggested Design	Comments
Pilot pharmacokinetic (single- and multiple-dosage)	I	Validation of analytical method; preliminary pharmacokinetics	Parallel groups (n = 36/group); suspension or solution dosage form if possible; minimal number (6/subject) of blood samples collected	Normally part of single- and/or multiple-dosage safety and tolerance studies
Pilot bioavailability	I	Pilot study to determine if solid dosage form adequate for phase II and III studies	Single-dosage crossover (n = 612); solution or suspension vs. solid dosage form	
Single-dosage ADME	I	Obtain definitive pharmacokinetic data, excretion pattern, dosage proportionality, definitive metabolism; projection of pharmacokinetic parameters for multiple dosing	Single-dosage parallel groups (6/group); radiolabeled drug in solution or suspension at two dosage levels; urine and feces as well as serial blood samples collected	
Intra- and intersubject variability	II	Determine PK variability expected during phase III pharmacokinetic studies and number of subjects required in future studies to assess bioequivalence	Three-way crossover (n = 12) using same dosage solution or suspension (dosages onehalf highest tolerated dose)	
Dose proportionality	II	Determine if bioavailability parameters (C^{max}, AUC) are linear over proposed dosage range	Three-way crossover (n = 12); solution or suspension covering the therapeutic range for a single dose	

(table continued on next page)

Table 2 Continued

Study	Clinical Phase	Objective	Suggested Design	Comments
Dosage proportionality	II	Determine if bioavailability parameters (C^{max}, AUC) are linear over proposed dosage range	Three-way crossover (n = 12); solution or suspension covering the therapeutic range for a single dose	
Multiple-dosage ADME	II	Validate single-dosage pharmacokinetic projections; determine ADME profile on multiple dosing; obtain large quantities of biological fluids for biotransformation studies	Single daily dose of radiolabeled drug in solution or suspension for 45 days in 6 subjects; C^{min} values obtained during dosing and complete blood profile after last dose; urine and feces collected during entire study	
Bioequivalence of service and proposed marketed dosage forms	III	Determine if dosage form(s) used during clinical trials is (are) bioequivalent to dosage form proposed for marketing; determine relative bioavailabilities of service and proposed market forms	Single-dosage crossover (n based on results of dosage proportionality and/or variability study using power calculation); highest strength dosage form proposed for market should be evaluated	Pivotal study for registratio
Dosage form proportionality	III	Determine if equipotent drug treatments administered as different dosage strengths of one dosage form produce equivalent drug bioavailability	Single-dosage crossover (n based on dose proportionality, variability, or prior bioequivalence studies); multiple strengths evaluated by bracketing (i.e., using lowest and highest dosage strengths for study)	

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Study	Clinical Phase	Objective	Suggested Design	Comments
Food interaction	III	Influence of food on bioavailability	Single-dosage crossover (n = 912); dosage form proposed for marketing should be used if available	
Effect of age	III	Influence of age on pharmacokinetic parameters	Single dosage in elderly volunteers (n = 25)	Results compared to those obtained from other pharmacokinetic studies using younger volunteers under the same experimental conditions
Renal insufficiency	III	Influence of renal function on pharmacokinetic parameters	Single dosage in patients (n = 12) with creatinine clearance <25 ml/min	Results compared to those obtained from other pharmacokinetic studies using subjects with normal renal function under similar experimental conditions; study not essential if renal excretion of drug and/or metabolites minimal
Hepatic insufficiency	III	Influence of hepatic function on pharmacokinetic parameters	Single dosage in patients (n = 12) with confirmed hepatic insufficiency	Results compared to other pharmacokinetic studies using subjects with normal hepatic function under the same experimental condition
Drug-drug interactions	III	Influence of concomitant medication on bioavailability parameters	Single-dosage three-way crossover (n = 1218); comparison is drug plus codrug, drug, codrug	Choice of codrug based on preclinical data, experience with other drugs, likely comedications

phase I to connote the original temporal definition for very early clinical development. Within each of three areas in this chapter, a discussion of general issues characteristically of concern at that juncture of development is followed by one or more specific examples of studies and methodological details. Most of the specific examples described in this chapter are based on development work performed at Sandoz Pharmaceuticals Corporation with the calcium channel blocker PN 200-110 (isradipine) [29], although studies with the drugs NB 106689 (fluperlapine) [30], OG 37325 (cyclosporin G) [31], cyclosporin A [31], HC 20511 (ketotifen) [32], and ZK 112119 (abecarnil) [33] are included when PN 200-110 data were unavailable or less suited to illustrate a particular point. Figure 1 shows the chemical structure of the compounds discussed in this chapter.

II. General Considerations

It is paradoxical that pharmacokinetic studies have become an integral part of the clinical development program for a new drug, in that the relationship between the time course of drug concentration in the compartments commonly measured and the therapeutic effect is usually complex and often defies rigorous quantification [3436]. This leads to the speculation that the utility of pharmacokinetic studies is less related to their own merits and more to the shortcomings inherent in the clinical evaluation process. Thus, regardless of whether a new therapeutic agent has been tested in a number of clinical trials, and regardless of the number of patients who have participated, the results cannot always be applied to the wider array of patients, clinical conditions, dosages, and durations of exposure that will be encountered when the agent is made available for general use [37]. Therefore, even though pharmacokinetic studies may only imperfectly explain the time course and dynamics of clinical effects caused by a new drug, they can contribute to the process of extrapolating clinical safety and efficacy data to the general population via bioavailability assessment and drug disposition studies in various groups and situations.

The design of a clinical pharmacokinetics study is determined by its objective and the clinical, bioanalytical, and statistical/mathematical tools available for its conduct and evaluation, within the constraints of ethical standards and availability of resources. The wide variety in the studies summarized in Table 2 requires consideration of many design factors, such as using one or multiple study centers, use of parallel, sequential (static vs. adaptive), or crossover study configurations, constant or variable dosing, blinded, unblinded, controlled (historically vs. within-study) or uncontrolled study format, use of normal volunteer or patient populations, and symmetrical or unbalanced blocking of study variables. These elements of clinical and clinical pharmacokinetics study design are excellently discussed in various texts (see for example [38,39]). The majority of clinical pharmacokinetic studies involve some crossover feature in which a subject

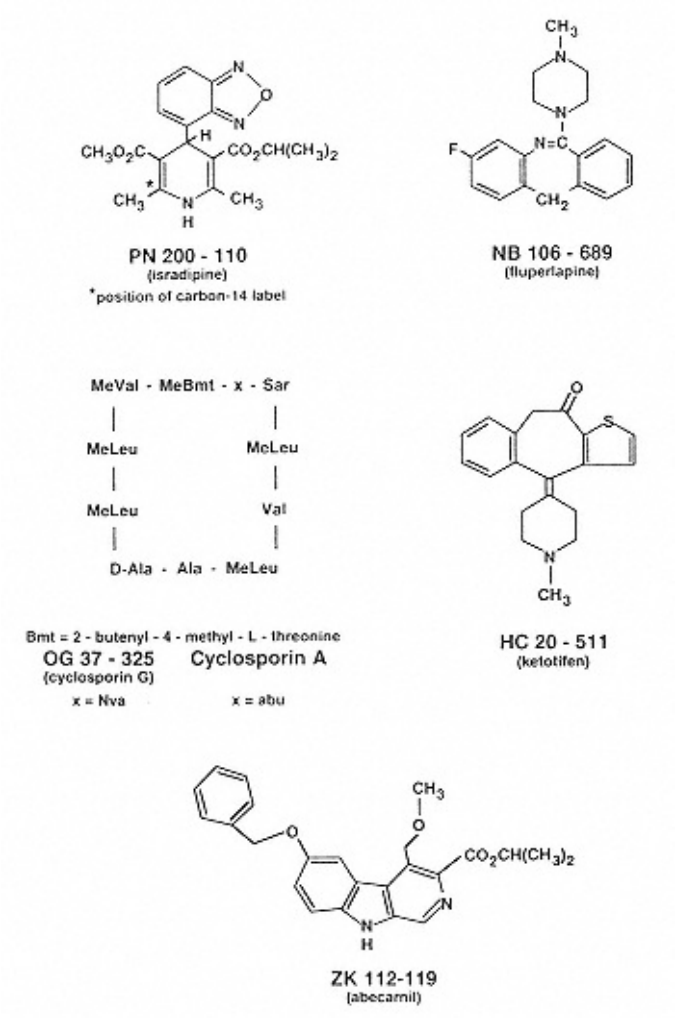

Figure 1

Chemical structures of compounds discussed in this chapter. PN 200-110 (isradipine) is a dihydropyridine derivative with vasodilating activity caused by inhibition of calcium movement through calcium channels [29]. NB 106689 (fluperlapine) is an experimental tricyclic neuroleptic agent [30]. OG 37325 (cyclosporin G [CyG], amino acid no. 2 = norvaline) is an analog of the cyclic undecapeptide cyclosporin A (CyA; amino acid no. 2 = aminobutyric acid) used clinically as an immunosuppressant [31]. HC 20511 (ketotifen) is an orally active antianaphylactic agent [32]. ZK 112119 (abecarnil) is a beta-carboline derivative developed as an antianxiety agent [33].

is his or her own control. However, the specific incorporation of this crossover design aspect into a final, statistically sound model, will depend upon the ability of the model to control for the variable(s) of interest. Analysis of variance is a well-established statistical method to estimate and test the effects and interactions of a number of experimental variables. Whenever appropriate, the design of a clinical pharmacokinetic study should be such to yield data suitable for this type of analysis (i.e., consistent with the statistical assumptions inherent in this type of analysis). Specific examples are provided below, particularly in Section III.C.3.

In the absence of safety concerns, the conduct of pharmacokinetic studies with normal, healthy volunteers has several advantages. Using such individuals can provide a larger data pool compared to patients because greater flexibility is allowed with respect to study entry criteria, scheduling of drug administrations, sampling of blood or other fluids, drug washout periods, changeover from one dosage form to another, and other such variables [28,40]. The categorical exclusion of women of childbearing age from volunteer studies [41] has given way to a case-by-case assessment of potential risk to the unborn when including women vs. the need to develop data in the female population, most particularly when the new drug is targeted for an indication primarily affecting women [41,42]. With a relatively homogeneous panel of normal volunteers, obtained by appropriate study entry restrictions with respect to gender, age, and other demographic variables, baseline pharmacokinetic parameters can be developed. The effect of other variables on the drug's pharmacokinetics, for example, disease state or specific organ function, can be evaluated against these baseline measures. As an alternative, comparisons of interest, such as the effect of a coadministered drug on the new drug's pharmacokinetics, can be performed under baseline conditions in a crossover design. Both approaches, in conjunction with a third, that of a general pharmacokinetic screen of the patient population used in the clinical trials, should contribute to the information needed for safe and effective general use of the new drug. Examples of the three approaches are cited in the latter parts of this chapter.

The success of a pharmacokinetic study in normal subjects or in patients depends to a large part upon careful preparation, including prior weighing of the risks and benefits of the study, elaboration of clear objectives, a thorough evaluation of all the various options for study design, and detailing the study objectives and methodologies in the study protocol. When executing the study, meticulous adherence to the protocol is mandatory. That portion of pharmacokinetic studies relating to safety should be evaluated by appropriate methods, which usually include physical examinations and determination of vital signs, electrocardiograms, and clinical laboratory parameters. Irrespective of the degree of safety of the drug to be administered, it is essential that the clinical portion of a

pharmacokinetic study be supervised at several levels of hierarchy and accountability. Negligence in seemingly unimportant details, even those unre-

lated to safety, can have serious repercussions. For example, a pharmacokinetic study can be compromised by easily avoidable problems, such as illegible or missing specimen labels or thawed specimens due to improper shipment from the clinical site to the analytical laboratory.

An additional factor for the successful completion of a pharmacokinetic study is the correct application of a well-characterized and validated bioanalytical method in generating drug level data, pharmacokinetic analysis and curve-fitting procedures appropriate for the data, and statistical techniques consistent with the type and volume of data generated under the study's specific objectives and design. The issues surrounding choice, development and validation of established or new bioanalytical methods, and their subsequent application to pharmacokinetic studies, have been discussed in detail [4345]. Validation of a method for its intended use, demonstration of acceptable performance during actual use, and confirmation of the stability of the analyte under the various storage and handling conditions of the pharmacokinetic study are prerequisites for scientific and regulatory acceptance of a pharmacokinetic study. Adherence to the principles and procedures of good laboratory practice is mandatory ([13]; also see Chapter 4 of this volume). Pharmacokinetic parameters can be derived from the drug concentration vs. time data via model dependent or independent approaches. Curve-fitting and pharmacokinetic modeling are well-accepted techniques for data evaluation, with simultaneous fitting of several compartments (e.g., blood, urine, and feces in absorption, distribution, metabolism, and excretion [ADME] studies as discussed below), providing meaningful model parameters. However, the model chosen should be the simplest configuration that can explain the available data. It is important that pharmacokinetic parameters, and in particular half-lives and area under the drug concentration vs. time curves, are derived from drug concentration data above the stated limit of quantification of the analytical method (as opposed to the limit of detection; see discussions in [4345]). This may not always be possible for pharmacokinetic evaluations of early phase I studies (see, for example, Section III.C.1) but is mandatory for bioequivalence and other significant pharmacokinetic studies (see, for example, Section III.C.3).

III. Clinical Phase I

A. Pharmacokinetics in the Selection of Clinical Dosages.

The primary purpose of phase I clinical studies is to obtain short-term safety data for a new drug, with preliminary efficacy data frequently obtained as a secondary objective. In an approach used widely by the pharmaceutical industry, the safety and tolerance of escalating single-dosage treatments of the new drug are established. This is followed by a

second study involving escalating multiple-

dosage administrations generally not exceeding 12 weeks per dosage level. These two studies normally use sequential groups of healthy normal volunteers, employing placebo control and double-blind conditions. However, alternative designs for the safety and toleration studies have been reported, which address the inherent inefficiencies of large subject panel size and time requirements of studies using sequential dose groups [28,46,47]. The initial dosage administered to humans normally represents one-tenth or less of the maximum justifiable dosage, which in turn is extrapolated from animal toxicity data. As outlined in the grey book, a monograph issued in 1959 by the Division of Pharmacology of the FDA, the rat should be able to tolerate without major toxicity ten or more times the maximum human dose and the dog a minimum of six times this dose on a milligram per kilogram basis. In the absence of more recent regulatory pronouncements, a safety factor of 10, 6, or 2 for rat, dog, and nonhuman primate, respectively, based on the species that was found to be most sensitive to the toxicological effects of the new drug, is normally used to determine the maximum justifiable human dosage. Developing appropriate factors is particularly difficult for drugs for which an exaggerated pharmacological response to the high dosages normally used in toxicity studies may obscure toxicological effects, or for which the pharmacological response may be mistaken for toxicity. On the other hand, absence of toxic findings at the highest dosage tested does not automatically justify using this dosage as the basis for safety factors. Toxicology studies should be designed to identify the toxic dosage threshhold, by increasing dosages until toxic effects occur and determining the highest dose showing no toxic effects. Even so, extrapolation of safety data from animals to humans based on the dosages administered is appropriate only if all of the species show similar absorption, bioavailability and biotransformation, as well as comparable sensitivity to the toxic effects of the drug or its biotransformation products. This is usually not the case. Thus, comparative pharmacokinetic and biotransformation data from all toxicology species can provide a means to assess the reliability of the extrapolation to humans. These data can be derived directly from the toxicology studies using toxicokinetic approaches [48] or can be obtained from separately conducted studies. If the new drug substance demonstrates poor, erratic, and/or dosage form-dependent oral absorption, nonlinear accumulation of drug or biotransformation products, unusual retention of drug or biotransformation products, an abnormal dose-response profile, or significantly dissimilar metabolite patterns between species, the maximum justifiable human dosage and, correspondingly, the initial human dosage should be lower than that extrapolated from the highest no-effect dose in the toxicity species. Various interspecies scaling techniques that take these pharmacokinetic issues into account have been applied to strengthen the extrapolations from animals to the human [4850] (also see Chapter 4). However, particularly for such problem drugs it is imperative that an evaluation of human pharmacokinetics during

clinical phase I be performed to corroborate the extrapolations. Based on a 1976 FDA recommendation [51] the following information should be routinely obtained in phase I:

1. Preliminary bioavailability and pharmacokinetic profile in humans: This provides drug exposure data that can be used to develop safety factors (based on AUC, C_{max}, or C_{min} comparisons) between the toxicology species and humans. These data also alert to potential bioavailability problems related to intrinsic drug properties or dosage form that could confound the evaluation of the clinical safety and subsequent efficacy studies.

2. Dose proportionality for single dosages: Such data serve as a basis for designing multiple-dosage regimens and may alert to potential safety or efficacy problems if nonlinearity is detected.

3. Preliminary drug metabolism in humans: Knowledge of the relative importance of various routes of drug elimination and the extent of biotransformation of the drug, as established by metabolite patterns, enhances interspecies comparisons of pharmacological effect and drug toxicity.

Some of these evaluations can be done as part of the usual single- and multiple-dosage safety and tolerance studies, although logistical factors and concerns that additional blood sampling may interfere with the clinical evaluations or compromise safety can either preclude this altogether or severely limit the number of samples that can be obtained. As an alternative, separate studies can be performed. The potential benefit of more detailed pharmacokinetic data normally provided by separate studies must be weighed against the greater expenditure in time and resources with this approach. In any event, studies utilizing radiolabeled drug, so called radioactive ADME studies, are conducted separately. As seen from the following examples, the complexity of the pharmacokinetic program and choice of separate or combined studies during clinical phase I should be determined by the considerations of safety, desired information, and available resources.

B. Pharmacokinetic Monitoring of the Phase I Safety and Toleration Studies

1. Single Escalating Dosage Study with OG 37325

In various animal models, the experimental drug OG 37325 (cyclosporin G, CyG) shows immunosuppressive effects equal to the structurally related marketed immunosuppressant cyclosporin A (CyA; see Fig. 1 for structures and abbreviations). CyG shows renal and hepatotoxic effects in the rat and other species at doses two- to threefold higher than the doses of CyA producing similar effects. However, the ratio of CyA dosage causing immunosuppression vs. renal toxicity is much lower in the animal models than is actually observed in humans

[31,52]. Therefore, despite a two- to threefold higher safety factor for CyG compared with CyA based on the animal data, the poor predictability of these data for the human raised concerns for CyG as well. These concerns regarding the initial administration of CyG to humans are reflected by the conservative phase I study protocol detailed below.

In a multiperiod, double-blind, randomized, placebo-controlled escalating dosage design, groups of eight subjects each (five active and three placebo) received single oral doses of CyG. The starting dosage was 1.25 mg/kg. CyA at this dosage shows no adverse effects. The next scheduled dosage (2.5 mg/kg CyG), as well as the subsequent dosages of 5, 7.5, 10, 15, and 20 mg/kg, were to be administered only if the safety of the previous dosage level had been ascertained based on clinical laboratory evaluations, medical examination, and complete CyG blood level profiles obtained by frequent blood sampling up to 72 h postdose. There were no significant findings of intolerability or adverse reactions towards CyG up to the highest single oral dosage of 20 mg/kg specified by the protocol. Transient elevations in liver enzyme levels were seen in four subjects in the active drug groups (5 and 7.5 mg/kg) and three subjects in the placebo group. Rechallenge of the subjects on active drug with the identical treatments, as well as the higher dosages of 10, 15, and 20 mg/kg administered to the subsequent groups, failed to elicit liver enzyme elevations. CyG blood concentrations were similar between subjects showing liver enzyme elevations and those with normal values. The occurrence of spurious changes in clinical laboratory parameters is a not infrequent problem requiring considerable resources for their evaluation and resolution. Drug concentration data can assist in determining their relevance. Blood concentrations of CyG in the low-dosage groups peaked (C^{max}) at 24 h postdose (t^{max}) and were <one-tenth of peak at 12 h postdose, but showed disproportionately lower peak and higher 12 h concentrations for the higher dose groups. However, the area under the concentration-time curve (AUC) increased in rough proportion to the dosage, as shown in Figure 2. There was considerable intersubject variation in the pharmacokinetic parameters (C^{max}, AUC, t^{max}) and in the terminal elimination half-lives, which showed a mean of approximately 11 h. Plasma concentrations of CyG, determined simultaneously from split blood specimens centrifuged at 37°C, were similar to the blood concentrations, irrespective of time postdose. In contrast, CyA blood concentrations are about twofold higher than the corresponding plasma concentrations. The previously mentioned serum liver enzymes, or other clinical and clinical laboratory data showed no relationship or trends with any of the CyG pharmacokinetic parameters.

The pharmacokinetic evaluation of this single dosage safety and toleration study with CyG provided support for escalating the dosages in the sequential groups, given the proportional, or less than proportional, increases in AUC and C^{max}, respectively. The

study provided data to support the twice-daily dosing

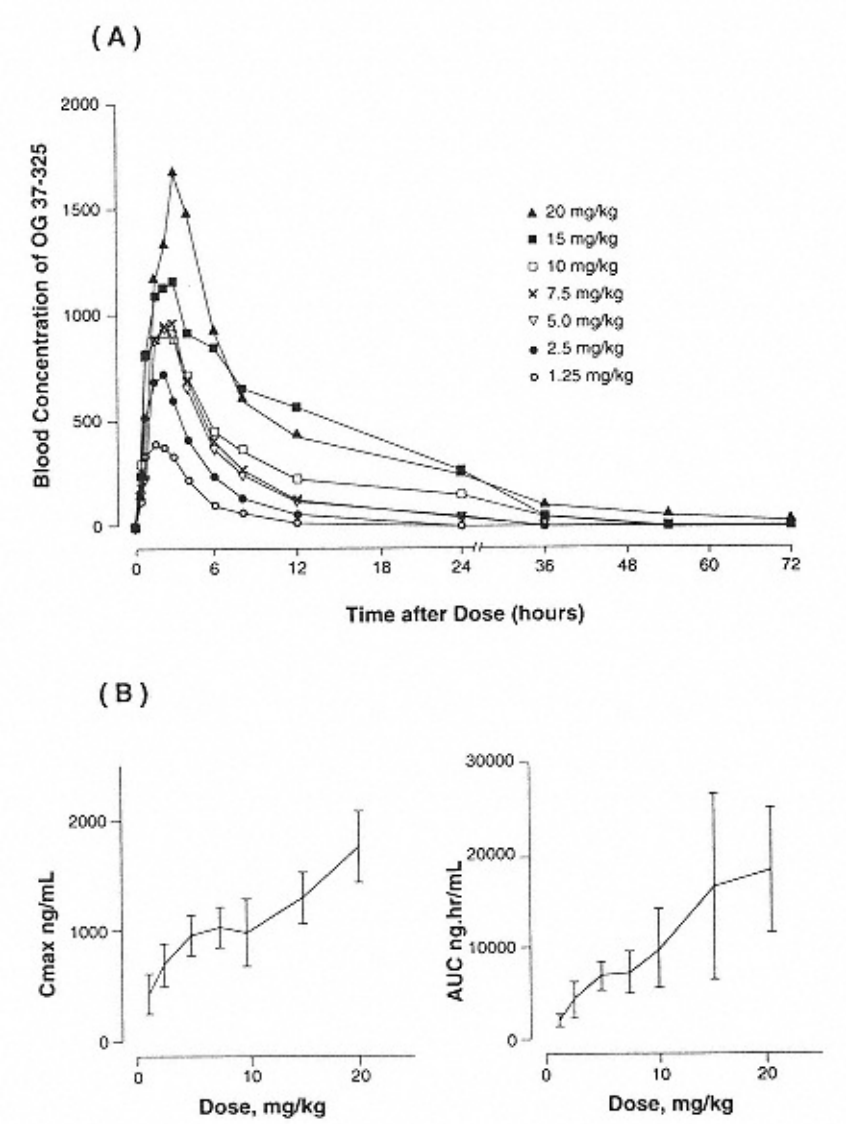

Figure 2
Mean blood levels (A) and derived pharmacokinetic parameters (B) of CyG in sequential groups of healthy normal volunteers administered escalating single oral dosages of CyG in a safety and tolerance study.

regimen and dosage adjustments in the subsequent phase I multiple dosage studies of CyG in patients with uveitis [53] and those who have received kidney transplants [54]. The single dose phase I study also yielded exposure data for comparison with the CyG blood concentration measurement in toxicity trials. The study revealed broad similarities in CyG pharmacokinetics with CyA, but also alerted clinicians to the lower distribution of CyG into the red blood cells compared to CyA. This study demonstrates that both individual and mean pharmacokinetic parameters obtained in the course of a single-dose safety and tolerance study can support the evaluation of drug safety. However, interpretation of the pharmacokinetic data and their relationship with dosage must take into account the variability inherent in intersubject and intergroup comparisons. A more rigorous assessment of the relationship of pharmacokinetics with dosage (dosage proportionality) is provided by a crossover design, as discussed in Section IV.A.2.

2. Steady-State Escalating Dosage Study with NB 106-689

For reasons of safety or practicality it may not always be possible to perform extensive drug concentration monitoring in subjects or patients enrolled in the early safety and toleration studies. For such drugs a more limited sampling scheme may have to be considered. The utility of incidental pharmacokinetic monitoring depends on the timing and frequency of sampling. The generation of complete drug level profiles at several dosage levels in the preceding example with CyG usually provides more detailed and reliable pharmacokinetic data than those obtained by occasional sampling. However, with judicious selection of sampling times for the drug under investigation, even limited blood sampling can yield useful information. The experimental drug NB 106-689 (fluperlapine; see Fig. 1) belongs to the class of tricyclic neuroleptic agents and therefore has the potential to affect adversely the hematopoietic system. Exposure of patients to prolonged high levels of the drug represents a risk factor, and of particular concern would be a disproportionate increase of drug level with increasing dosages. Early generation of drug level profiles and determination of pharmacokinetic parameters would be advantageous for this type of drug; however, frequent blood sampling is undesirable as part of a safety study in which the effects on the hematopoietic system are monitored. Although measuring urinary concentrations frequently represents an alternative approach, this was not considered appropriate for NB 106-689 since preclinical studies showed that the major route of elimination of the drug is in the bile. As a consequence, in the multiple-dose safety and tolerance study of NB 106-689, a limited number of plasma samples for drug level analysis were obtained for each dose group. Five groups of 10 normal volunteers (8 active and 2 placebo) each received two or three daily doses of drug ranging from 30 to 200 mg/day for a period of 7 days. Progression to the next higher dosage group occurred only upon evaluation of all safety data

for the previous group. Blood for clinical laboratory evaluations was obtained during the baseline (predose) stage of the study, on the morning of dose day 3, on the morning of dose day 7, and during the washout phase of the study (day 10). Plasma levels of NB 106-689 were determined from aliquots of these blood specimens. The results are summarized in Figure 3. Irrespective of the dosing day, there was a linear relationship between the C_{min} values and the dosage. Based on the increase in drug levels between days 3 and 7 and the decline in drug levels between days 7 and 10, a terminal elimination half-life of about 20 h could be estimated for NB 106-689. This was confirmed in subsequent studies.

With a drug showing a substantially shorter half-life of elimination than NB 106-689, monitoring of the trough levels may be inappropriate. In such a case,

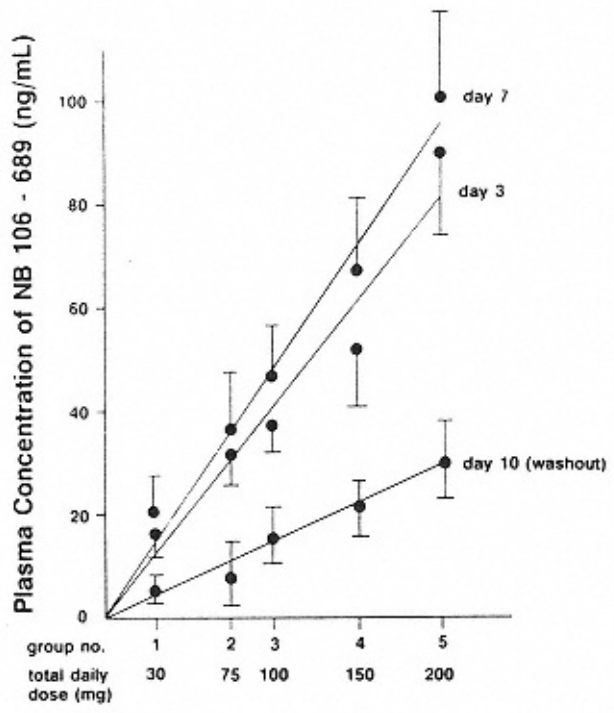

Figure 3
Mean plasma levels (±S.D.) of NB 106-689 in groups of normal volunteers during the course of a 10 day regimen of the drug involving various dosage levels: group 1, 10 mg 3 times a day; group 2, 25 mg 3 times a day; group 3, 50 mg twice a day; group 4, 50 mg three times a day; group 5, 100 mg twice a day. Solid lines represent linear regression equation through the origin.

drug concentration monitoring over 34 dosing days fixed at such time point postdose at which adequate drug levels are expected to occur should provide information concerning possible nonlinear accumulation of drug. However, the data must be viewed with caution, given the higher variability in the drug concentrations expected from such a sampling scheme.

C. Performance of (Pilot) Bioavailability and Single-Dosage ADME Studies

As shown by the above examples, monitoring of the single dosage and multiple dosage safety and toleration studies can provide useful, although limited, pharmacokinetic data. The following, separately conducted, phase I studies with the drug PN 200-110 (isradipine; see Fig. 1), demonstrate that such studies can provide additional information and substantially increase the quality of pharmacokinetic information obtained. The first time in humans study with this antihypertensive agent assessed the safety and toleration of PN 200-110 in a randomized, double-blind, parallel group, placebo-controlled design. The single oral doses administered ranged from 2.5 to 20 mg, with 20 mg or about 0.4 mg/kg representing one-tenth of the highest no toxic effect dosage of 4 mg/kg in the dog, the species most sensitive to the adverse effects of PN 200-110. Significant mean decreases in blood pressure were observed in the volunteers at the 10, 15, and 20 mg dosages, the effect being greatest with the highest dosage. Single oral doses of 15 mg or less were better tolerated than the 20 mg dose [29,55]. Instead of direct monitoring of this phase I study to evaluate the relationship among drug dose, plasma level, and effect, early pharmacokinetic information on PN 200-110 was obtained from two separate studies, a bioavailability and an ADME study whose protocols were not hampered by the double-blind controls, presence of placebo-treated subjects, and the limitations in blood sampling frequency posed by the safety and tolerance study.

1. Pilot Pharmacokinetic and Bioavailability Study with PN 200-110

In this study, six normal healthy male volunteers each received single oral doses of PN 200-110 on six separate occasions as 5 and 20 mg clinical service capsules, 5 and 20 mg as an oral solution, and 5 and 20 mg in solution administered sublingually. Multiple blood samples were obtained at fixed time points up to 24 h postdose. PN 200-110 plasma concentrations were analyzed by a high-performance liquid chromatography (HPLC) method developed earlier for the pre-IND animal studies. The following key information was obtained from this pilot pharmacokinetic study: there exists considerable intersubject variability for PN 200-110 bioavailability parameters AUC, C^{max}, t^{max}, and rate constants for absorption and the biphasic elimination; the oral and sublingual routes of administration yield a similar rate and extent of bioavailability; the capsule formulation showed reduced AUC compared with the solution and a C^{max} about

one-half that of the solution; PN 200-110 AUC and C_{max} show rough dosage proportionality irrespective of formulation; and plasma concentrations of PN 200-110 were in the low ng/ml range and the elimination of drug from plasma is rapid. The following conclusions and consequences for further development of PN 200-110 resulted from this pilot study. First, the sublingual route of administration is not relevant for PN 200-110; however, additional formulation work would be required to improve the capsule. Second, improvements in the analytical methodology were necessary to measure accurately and reproducibly the low plasma levels encountered. Third, from the plasma level profiles and drug half-life a twice a day or more frequent dosage regimen would be appropriate provided that the suggestion of a direct correlation between drug level and pharmacological activity in this normotensive volunteer study could be confirmed in hypertensive patients.

The pharmacokinetic data of this pilot crossover study, in conjunction with the safety, tolerability, and pharmacodynamic data from the escalating single-dosage trial in parallel groups of subjects, provided valuable information at an early stage of drug development concerning effective drug level, possible dosage regimens, and dosage form performance for PN 200-110, and triggered improvements in the bioanalytical methods available for this drug [56,57]. However, it is clear that such exploratory or pilot pharmacokinetic programs must be tailored to the situation and requirements posed by each new drug substance. In particular the crossover design involving six separate PN 200-110 treatments may be too ambitious for some drugs, leading to a high dropout rate and other complications. Several smaller studies each involving a three-way crossover, could provide the same information at less risk. Therefore, this pilot pharmacokinetic study with PN 200-110 serves only as an illustration of one of many possible approaches toward pharmacokinetic activities in early clinical phase I. The approach taken may or may not include pilot studies, but should involve at a minimum the single-dosage ADME study described below.

2. Single-Dosage ADME Study (Radioactive) with PN 200-110

Preclinical pharmacokinetic studies showed that PN 200-110 is subject to considerable first-pass metabolism [58]. First-pass metabolism is frequently associated with variable and nonlinear bioavailability and pharmacokinetics. Despite the high variability of bioavailability parameters seen in the pilot pharmacokinetic study, there was an indication of dosage proportionality in the 520 mg range, which suggests the absence of saturable pharmacokinetic processes for PN 200-110. Nevertheless, a more rigorous assessment of first-pass metabolism and potential nonlinearities was desirable, by conducting an absolute bioavailability study, a more definitive dose-proportionality study (both

discussed in Section IV), or a single-dose (radioactive) ADME study.

Although single-dose human ADME studies are relatively simple in design, usually employing no more than 612 subjects, if performed judiciously they can provide a wide range of useful information, additional to assessment of firstpass metabolism. The objectives of such studies should include the following:

1. Estimation of the rate and extent of absorption of the administered dose: Since total radioactivity is measured, one can be assured that unchanged drug and all metabolites (total dose) are included, provided the radiolabel has been appropriately positioned (not metabolically labile).

2. Determination of the rate and amount of elimination from the blood as well as the excretory pathways (e.g., urine and feces): Since various analytical techniques readily allow differentiation of parent drug and metabolites in the radioactive sample, conclusive information on the fate of the drug in humans can be obtained. Knowledge of the contribution of renal and hepatic pathways to the elimination of the drug and its metabolites is important for the use of the drug in certain disease states (e.g., renal or hepatic insufficiency; see Sections V.C.2 and V.C.3).

3. Provide data on metabolites and biotransformation pathways: Biotransformation studies on new drugs are normally performed with radioactive samples. Thus blood, urine, and feces from human ADME studies provide a source of biological materials for biotransformation work. Using radiolabeled drug greatly facilitates the determination of metabolite patterns as a function of dosage and time postdose. Such patterns are obtained by HPLC of the biological fluid and monitoring of the radioactivity in the eluent. Isolation of the appropriate peaks allows further purification and structural characterization and identification of metabolites.

4. Generation of pharmacokinetic parameters for both parent drug and metabolites: This information can be employed to design dosing regimens and sampling times for future clinical trials.

5. Provide definitive data on material balance: Since this type of study can yield complete accountability of the administered dose, information concerning retention and/or accumulation of drug and metabolites can be obtained.

6. Provide data that are directly comparable to those generated in the preclinical animal ADME studies: This allows the development of rational animal models.

Human ADME studies are usually performed in normal, healthy, male volunteers. A single dose of the radioactive drug is administered, and if more than one dosage level is to be investigated, parallel groups are utilized. Since radiolabeled material (usually carbon

14 or tritium) is administered in these studies, it is necessary to evaluate the potential hazard of radiation exposure to the volunteers. Of particular importance is what is referred to as the absorbed

dose of radioactivity, which is normally determined by estimating the energy (rad or rem) imparted to a specific organ or tissue. This can conveniently be estimated by the following equation [59]:

$$D = 73.8\bar{E}_{\beta}C_{max}T_{eff}$$

where D = absorbed dose (rad or rem), $\bar{E}_{\beta}$ = average b-particle energy (MeV), C_{max} = maximum tissue or organ concentration (μCi/g), and T_{eff} = effective biological half-life (days).

For example, if 250 μCi of carbon 14-labeled PN 200-110 with an assumed terminal half-life of 48 h for the radioactive label were to be administered to a volunteer, the exposure to the liver (based on a 310 g mass in a 70 kg man) would be calculated to be

$$D = (73.8)\ (0.05\ MeV)\ \frac{250\ \mu Ci}{310\ g}\ (2\ days) = 5.95\ rem$$

This value, almost twice the allowable limit for a single dose [60], is an unrealistic estimation since it assumes that the entire radioactive dose localizes in the liver with none distributing to other organs or tissues of the body. A more realistic approach is the use of in vivo animal distribution data in which actual organ and tissue concentration of radioactivity is determined following administration of the labeled drug. For PN 200-110, a maximum radioactivity concentration of 2.25 μgEq/g was observed in the liver of rats following a 0.25 mg/kg oral dose of the ^{14}C-labeled drug. Thus the estimated maximum liver concentration following a radioactive dose of 250 μCi in a 70 kg man would be

$$C_{max} = \frac{2.25\ \mu gEq/g}{0.25\ mg/kg}\ \frac{250\ \mu Ci}{70{,}000\ g}$$

The projected exposure of radioactivity to liver in humans would be

$$D = 73.8(0.05\ MeV)(0.032\ \mu Ci/g)(2\ days)$$

$$= 0.236\ rems$$

This value is approximately 25 times less than that calculated without the benefit of the animal tissue distribution data and is considerably less than the allowable 3 rem limits for organs of this type [59,60]. Similar exposure data should be generated for other critical organs and tissues, since in the absence of animal distribution data an extreme overestimation of radioactivity burden normally occurs.

Although the radioactive dose is often administered orally as a solid dosage form (tablet or capsule), a solution or suspension is preferable. In this manner the pharmacokinetics of the drug is not obscured by biopharmaceutical factors,

such as disintegration or dissolution of the dosage form. However, appropriate stability data for the solution must be generated prior to the study.

In the usual study the radiolabeled dosage form is administered to volunteers after an overnight fast. Serial blood, urine, and fecal samples are then collected for a sufficient time to ensure that essentially all of the radioactive dose can be accounted for. In normal cases, 120 h or more may be required since in this type of study the radioactive samples contain not only the parent compound but also all metabolites bearing the radiolabel. If it is suspected from preclinical investigations or from the position of the label per se that biological instability of the label could occur, the study design should include either the collection of carbon dioxide (exhaled air, ^{14}C label) or analysis for tritiated water (urine, ^{3}H label).

The phase I single-dosage ADME study involved administration of radiolabeled PN 200-110 to two parallel groups of normal healthy male volunteers [55]. One group of six volunteers received a 5 mg dose, the other a 20 mg dose. The PN 200-110 used in the ADME study was radiolabeled with carbon 14 in the 2-position, as shown in Figure 1. This position is stable to hydrolytic and biotransformation processes, as demonstrated in preclinical animal studies by the absence of exhaled radiolabeled carbon dioxide and complete recovery of radioactivity from the excreta. Analysis for unchanged [^{14}C]PN 200-110 in blood was done by radioimmunoassay [57] and by HPLC coupled with radioactivity monitoring. In the latter, quantitation of the low levels of PN 200-110 was achieved by addition of nonlabeled PN 200-110 to the specimen and counting the radioactivity present in the elution window described by the ultraviolet (UV) trace of the added drug [61]. Direct quantitation of parent drug in the biological fluid is possible by addition of large quantities of nonlabeled drug, extraction, and recrystallization to constant specific activity (inverse isotope dilution).

Figure 4 depicts the mean blood levels and the cumulative excretion of [^{14}C]PN 200-110 following the 5 mg oral dose. Qualitatively similar results were obtained for the 20 mg dose of [^{14}C]PN 200-110. At both dosages the recovery of radioactivity was nearly complete. Incomplete recovery of radioactivity can jeopardize the validity of an ADME study, and thus efforts should be expended to determine if this was due to incomplete collection, losses through specimen workup or as volatile radioactivity, or was caused by the retention of radioactivity in the body. If the last was found to occur, an investigation of the mechanism of this retention is mandatory. Enterohepatic or other modes of recycling, storage in a deep compartment, or actual covalent linkage to tissue are examples of processes leading to drug or metabolite retention.

The single-dose ADME study with [^{14}C]PN 200-110 confirmed the finding of dosage

proportionality in the pilot study described in Section III.C.1, as well as the existence of a high first-pass effect, previously seen in animals.

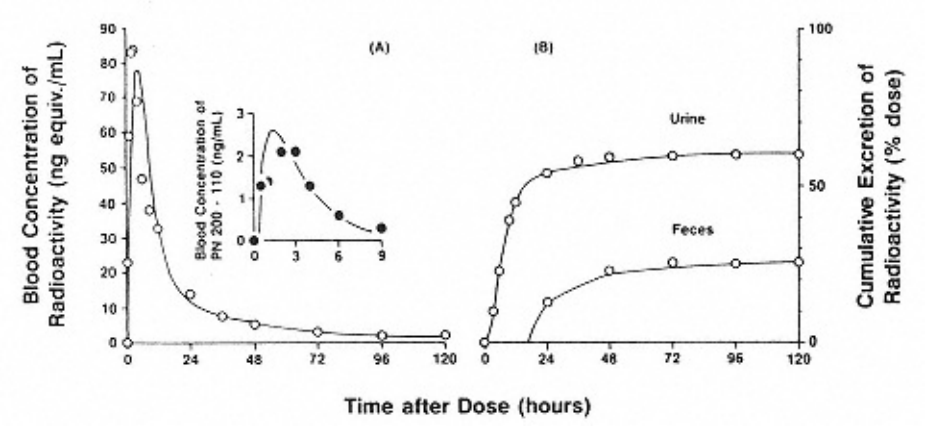

Figure 4
Mean blood levels (A) and cumulative excretion (B) following a 5 mg oral dose of [14C]PN 200-110 to six normal, male volunteers. ○, 14C concentrations; ●, PN 200-110 concentrations. The curves are computer derived. Excretion of unchanged drug in the urine and feces was <10% of dose.

Through the use of an appropriate model involving simultaneous fitting of all data points from the three compartments (blood, urine, and feces), single-dose pharmacokinetic parameters could be calculated and multiple-dose projections made. This is discussed in Section IV.A.1, in which simulated curves based on the single-dose ADME data for PN 200-110 are compared to the actual concentrations of radioactivity and unchanged drug resulting from a multiple-dose study in normal male volunteers performed during clinical phase II of the PN 200-110 development program (also see Table 2).

3. Pilot Bioequivalence Study with ZK 112119

The range of doses explored in the early safety, toleration, and preliminary efficacy trials can be quite large, as exemplified by the study with OG 37325 discussed in Section III.B.1, with the range between the starting dose of 1.25 mg/kg and the highest dose of 25 mg/kg representing a factor of 20. The range between the lowest and highest dose in safety and toleration studies often exceeds a factor of 10, and may on occasion exceed two orders of magnitude. This adds to the difficulties of preparing suitable dosage forms. A further challenge to the development of clinical service forms and dosage strengths is that at this early stage there may exist limited information on the NCEs physicochemical properties, its compatibility with and stability in the presence of excipients, and the biopharmaceutical consequences of specific properties of the formulation and its in vitro dissolution profile. Drug solutions, such as the corn

oil formulation used for OG 37325 (see Section III.B.1), provide greater flexibility in dosing, but at the cost of less convenience, potential stability issues, and, as discussed in Section V.B, a reduced likelihood of developing solid follow-up dosage forms that are bioequivalent to the solution.

The early clinical experience with ZK 112119 (abecarnil; see Fig. 1) prompted exploration of a wider dosage range and higher dosages than envisaged, which made it desirable to complement the available 1 mg capsule strength with a 2.5 mg capsule. The proposed use of this 2.5 mg capsule in pivotal phase II dosage ranging investigations called for demonstration of bioequivalence of the 1 and 2.5 mg ZK 112119 capsules (e.g., equipotent doses of the two capsules, both being of the same size but differing in the ratio of active to inactive ingredients), which should yield identical plasma concentrations of ZK 112119. This is referred to as dosage form proportionality.

Guidelines for the basic design and conduct of bioavailability and bioequivalence studies for new drug products or new formulations of active drug ingredients already approved for marketing are provided in 21 CFR 320.25 [8]. Additional, detailed guidelines focusing on the two-period, two-treatment, 2 × 2 crossover study have been elaborated by the FDA's Office of Generic Drugs [21]. This study design, frequently used for comparing a generic formulation of an approved drug with the innovator's formulation, was applied to the bioavailability assessment of the two ZK 112119 capsules.

In an open-label randomized two-way crossover study, 12 normal healthy male subjects received single oral 5 mg doses of ZK 112119 as 5 × 1 mg or 2 × 2.5 mg clinical service capsules. A period of not less than 7 days was observed between the two drug administrations. This ensured a washout of ZK 112119 (half-life of approximately 10 h), as well as its metabolites (total radioactivity half-life for [^{14}C]ZK 112119 of 22 h [62]). Plasma concentrations of ZK 112119 were determined to 36 h postdose using a validated HPLC method [4143]. Prior to all calculations, which included trapezoidal assessment of AUC 036 h, the ZK 112119 concentrations below the limit of quantification (LOQ) were set to zero, which is analogous to the guideline procedure of determining trapezoidal AUCs to the last time point above the LOQ [21]. A triexponential expression including a lag time to absorption [63] was used to curve-fit the plasma concentrations of ZK 112119 using the nonlinear least square regression program (NONLIN) [64]. This allowed calculation of interpolated t_{max} and C_{max}, AUCs to infinite time, and half-lives. Curve-fitting and modeling of data using NONLIN or other approaches are not mandatory for bioequivalence studies, but can provide useful insights regarding pharmacokinetic properties of a drug that can be supportive of the bioequivalence objective. The statistical evaluation of the parameters, derived from raw and curve fitted data, was done using

analysis of variance.

The FDA specifies analysis of variance to test for treatment differences and indicates that the following assumptions must be met to validate use of the analysis of variance [21]:

The subjects chosen for the study should be randomly assigned to the sequences of the study: Appropriate randomization procedures are fundamental to the interpretation of statistical data. The use of a Latin square design for a balanced crossover study can offer optimal combinational properties for the by column and by row arrangement in the square of the fixed and random study variables being examined (e.g. drug treatment, study period, subjects, and sequence of administration) [65]. This allows efficient analysis for these variables, particularly if the within-subject variability is small relative to the between-subject variability. The treatments for the 12-subject ZK 112119 study were assigned by six 2 × 2 Latin squares, using the same formal randomization procedures as employed for higher order (e.g., 3 × 3 and greater) Latin squares [66]. The absence of a carry-over effect, an unlikely occurrence due to the long washout period observed, was confirmed by no detectable ZK 112119 concentrations in the subjects' predose plasmas during study period 2.

The variances associated with the two treatments, as well as between the sequence groups (e.g., 1 × 5 mg, then 2 × 2.5 mg capsule = sequence 1, 2 × 2.5 mg capsule, then 1 × 5 mg capsule = sequence 2), should be equal or at least comparable: This can be tested statistically [67] and was found to be nonsignificant for the ZK 112119 study.

The main effects of the statistical model, such as subject, sequence, period, and treatment should be additive and show no interactions among each other: The analysis revealed no interactions.

The residuals of the model should be independently and normally distributed: To ensure normal distribution of the bioavailability parameters, logarithmic transformation prior to statistical analysis is encouraged [21]. Log transformation is especially desirable when the number of study subjects is relatively low, as in this ZK 112119 study, or when an unequal number of subjects per treatment is encountered due to dropouts or other reasons. This latter situation also necessitates an alternative analysis of variance approach, in which the data are grouped as randomized blocks, with an attendant loss of precision [66].

Analysis of variance was used to evaluate the critical parameters AUC, C_{max}, and t_{max}. The statistical power to discern 20% differences in parameter means at the a = 0.05 level was calculated. The two one-sided t-tests procedure as applied by Schuirman [68], the symmetrical 95% confidence limit approach developed by Westlake [69], and the conventional 90% confidence limits for ratio means [21] were used for the bioavailability

comparisons. Although the 90% confi-

dence limits for the logarithmic transforms of the data represent the principal regulatory criterion for judging bioequivalence, the other calculations should be done to provide corroborative information. The results of these comparisons are summarized in Table 3. Bioequivalence by strict regulatory standards, with the 90% confidence interval falling within the range 0.81.25 [21], was achieved for AUC only. Curve-fitting to yield interpolated C_{max} and t_{max} did not improve statistical acceptability for these parameter comparisons. As shown by C_{max}, the confidence band criteria for bioequivalence are more discriminating than the approach declaring bioequivalence if differences in the means are <20% and there exists adequate power (e.g., ≥0.8) to detect 20% differences.

The presence of outliers, such as subject 4 in the ZK 112119 pilot study, can adversely affect statistical outcome, exemplified by the individual and mean plasma concentration curves and C_{max} ratios shown in Figure 5. With subject 4 removed, the statistical analysis showed bioequivalence for C_{max}. However, in the absence of compelling clinical or other type of evidence, identification of subject 4 as an outlier by inspection or even on the basis of statistical tests [70] would not be sufficient grounds for removal from the statistical analysis.

The observation of t_{max} differences between the two ZK 112119 capsules should not be relevant for this chronically administered anxiolytic agent. Administering multiple capsules per dose is not optimal for determining exact t_{max} values, due to variability in absorption of individual capsules and may have contributed to the observed variability in t_{max} (see Table 3). However, the alternative approach of single-capsule administration and statistical analysis of t_{max}, and of dose-normalized AUC and C_{max}, would have to be based on the, at that stage uncertain, assumption of dosage proportionality (demonstrated in a later study). This approach also could have encountered bioanalytical difficulties owing to the low plasma concentrations expected from a 1 mg dose.

Although this study met its objective of demonstrating acceptable bioavailability performance of the 2.5 mg ZK 112119 capsule for later phase II trials, it did not provide proof of bioequivalence and raised the potential of product failure or unusual drug disposition from the deviant results shown by subject 4. His plasma concentration curves were substantially lower than the study mean, particularly in one of the study periods (see Fig. 5). Additional investigations conducted in phase II and III to explore these issues showed no evidence of product failure and revealed no subgroup among young white males with significantly different pharmacokinetic profile of ZK 112119 (see Section IV.B). This left misdosing (subject taking fewer than the assigned capsules or tablets either deliberately or through a dosing error) as a reasonable explanation, and demonstrates the need to ensure the validity of a clinical pharmacokinetic study by

carefully attending to all details and checking all critical steps, such as drug dosing, blood sampling etc. (see Section II). It also points out a frequent dilemma during drug development, of having to base important decisions such as

Table 3 Descriptive Statistics and Treatment Comparisons of Bioavailability Parameters of 1 mg and 2.5 r

Parameter	Arithmetic Mean ± SD Geometric Mean (Range) 5 × 1 mg Capsule	2 × 2.5 mg Capsule	% Difference	Significance	Power to Detect 20% Differences in the Means	Percentage of Subjects with Ratios of 0.751.25	Schuirmann's Two One-Sided T-Tests
Raw Data							
AUC (036 hr)	69.3 ± 37.5	65.3 ± 34.9	6.1	NSa	.99	83	b
(ng.h/ml)	59.7 (20.9121.7)	55.1 (14.1116)	8.4	NS			
Cmax	8.03 ± 2.66	7.79 ± 3.77	3.0	NS	.84	67	b
(ng/ml)	7.64 (4.8412.9)	6.86 (1.8615.4)	11.3	NS			NSd
tmax	1.60 ± 0.47	2.00 ± 1.09	-19.8	NS	<.50	25	NS
(h)	1.53 (0.752)	1.77 (14)	-13.9	NS			NS
Curve-Fit Data							
AUC (0∞)	78.0 ± 44.3	73.3 ± 43.6	6.4	NS	.99	67	b
(ng.h/ml)	66.2 (22.2153)	59.9 (15.3157)	10.5	NS			c
Cmax	7.85 ± 2.83	7.52 ± 3.71	4.3	NS	.80	67	c
(ng/ml)	7.38 (3.9713.2)	6.60 (1.8315.0)	11.8	NS			NS
tmax	1.62 ± 0.457	1.89 ± 0.90	-14.4	NS	<.50	33	NS
(h)	1.56 (1.002.34)	1.73 (1.103.71)	-9.9	NS			NS

aNS, not significant, $p > 0.05$.

bsignificance, $p < 0.05$ for both tests; bioequivalence was achieved.

c$0.05 < p < 0.1$ for both tests; borderline bioequivalence.

dNS $p > 0.1$ for both tests

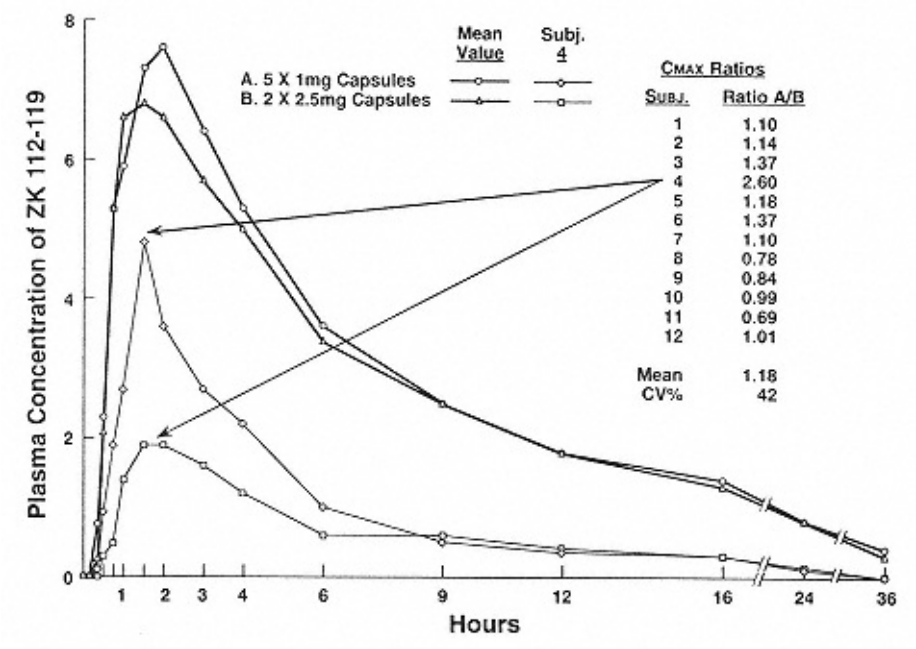

Figure 5
Mean plasma levels of ZK 112119 after administration of a 5 mg dose as 5 × 1 mg capsules ○ or 2 × 2.5 mg capsules △ compared to plasma levels of outlier subject showing extreme Cmax ratio.

whether or not to proceed to clinical phase II with a particular service form on incomplete or less than rigorous data, the alternative being to repeat the study with its attendant costs in resources and time.

IV. Clinical Phase II

A. Profiling Pharmacokinetic Performance

During this phase of drug development, the first trial of the new drug in patients normally occurs. The primary objectives are to evaluate the efficacy of the drug and to establish its safety under conditions approaching the intended use. This usually involves multiple-dose regimens administered to patients over an extended time period from which sufficient efficacy and safety data are obtained to justify entering the long-term trials of clinical phase III. Pharmacokinetic activities in clinical phase II support two general areas. Support of clinical studies includes more detailed investigations of drug pharmacokinetics and dosage

form performance, especially under conditions of multiple dosing, in normal volunteers but also in patients if there is reason to believe that drug disposition in the latter may be significantly different. Support of the long-term animal safety studies includes a more complete evaluation of animal and human metabolism, such as metabolite characterization through metabolite patterns, isolation of major metabolite(s) from appropriate biological fluids, and structural characterization and/or identification. A comparison of the type and relative importance of biotransformation pathways in animals and humans validates the choice of the species for long-term toxicity testing, which is normally initiated at this stage.

1. Multiple-Dosage ADME Study with PN 200-110.

When substantially long half-lives of radioactivity are observed during the single-dosage ADME study (Section III.C.2), it is desirable to conduct a multiple-dose radioactive ADME study (see Fig. 6). In addition to providing multiple-dosage pharmacokinetic data for both parent drug and total radioactiv-

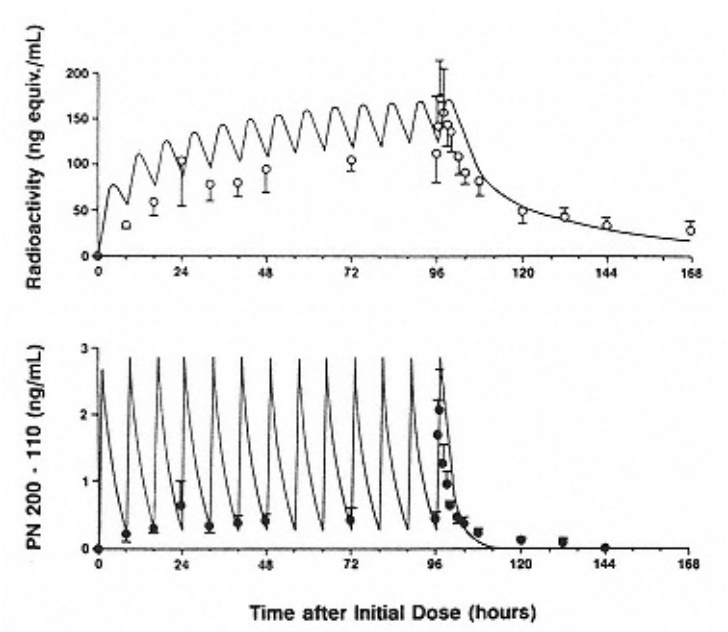

Figure 6
Mean blood levels of (○) radioactivity (ng Eq/ml) and (●) PN 200-110 (ng/mL) during multiple oral administration (5 mg every 8 hr × 13) of [14C] PN 200-110 to five normal male volunteers. Bars indicate 1 S.D. The curves are simulated based on single-dosage parameters.

ity and supplying mass balance data, the study also ensures that there are no accumulating metabolites that would not be detected following a single dose. Of special interest is an assessment of whether linear pharmacokinetic processes (additivity principle) can account for the accumulation of parent drug and metabolites from single-dosage to steady-state concentrations. The single-dosage ADME study data can be used for such extrapolation and comparison. The design of the multiple-dosage ADME study should ensure achievement of steady-state conditions for parent drug and radioactivity. Samples for trough concentration determinations should be collected throughout the study. Complete urine and fecal collections, which allow mass balance determinations and serve as bulk source for isolation of metabolites, should be considered for a minimum of 3 days at steady state. This allows calculation of the average elimination of drug into the urine or feces over a 24 h period or the relevant dosing interval. The results of the multiple-dosage ADME study with PN 200-110 showed linear pharmacokinetic processes for parent drug and radioactivity (Fig. 6), and a fecal/urine excretion ratio of about 2:1, as were also observed in the single-dosage ADME study.

There is some question as to the optimal dosage form for the multiple-dosage ADME study. Using a solution provides comparability with the single-dosage ADME study. However, unless solutions are freshly prepared prior to dosing, the chemical and radiochemical stability of the drug in solution over the entire study period has to be established. Using the clinical service form (the dosage form chosen for the extended clinical trials in late phase II and in phase III) for the radioactive dose provides steady-state kinetic and mass balance data more representative of the clinical situation. Such data could be very useful to support clinical evaluations, complementing the phase II population pharmacokinetic and pharmacodynamic investigations (see Section IV.B). Practical considerations, such as small batch size and the safety issues raised by introducing radioactive drug substance into the pharmaceutical production equipment, may preclude the manufacture of radioactive clinical service forms. Therefore, finely packed capsules prepared by hand in a suitably restricted environment or, preferably, a solution, are normally used for the radioactive ADME study. The evaluation of dosage form performance of the clinical service form can be accomplished by separate studies using normal volunteers such as the pilot ZK 112119 study discussed in Section III.C.3, or by monitoring drug concentrations in the phase II studies in patients. The choice of either approach, special studies in volunteers vs. monitoring patients, and the extent of such monitoring should be guided by the considerations already discussed for clinical phase I.

Evaluations of the dosage form and dosage strengths to be marketed should also be initiated at this juncture, particularly if the clinical service form is not a likely candidate for marketing. Early scheduling of activities is imperative because of the extended time

requirements for developing, and in particular

stability testing, the new dosage form. In the event that the clinical service form is found to be not bioequivalent to the dosage form proposed for marketing, sufficient time for development of an alternative form should be available prior to the targeted NDA date. Even when the clinical service form is intended for marketing, early bioavailability testing is desirable to ascertain its acceptable performance compared with a solution or suspension of the drug (the ideal formulation). Suboptimal bioavailability increases the potential for higher inter- and intrasubject variability and should be avoided. If multiple strengths of the clinical service form are to be marketed, their therapeutic equivalence will have to be demonstrated in a dosage form proportionality study (see Table 2 and Sections III.C.3 and V.B), unless therapeutic equivalence can be established clinically or all dose strengths use the same excipients and have identical ratios of active to inactive ingredients. If the final choice of dosage form to be marketed cannot be made at this stage, prudence dictates that all work necessary for drug registration (i.e., NDA) be completed as far as possible prior to the ultimate decision point. This may include a dose-proportionality study and an intrasubject variability study, examples of which are discussed in the following sections (also see Table 2) and may also involve attempts to correlate the in vitro dissolution rate with bioavailability in pilot studies.

2. Dosage-Proportionality Study with PN 200-110

The objective of the dosage-proportionality study is to determine the pharmacokinetics of the new drug over the entire dose range employed in clinical use. The effect of dosage on the rate and extent of the bioavailability of the new drug, as reflected by AUC, C_{max}, and t_{max}, are established. Other pharmacokinetic parameters based on model dependent or independent analysis of the drug concentration vs. time data can also be evaluated. For example, mean residence time may provide more insight than do C_{max} and t_{max} alone regarding the input kinetics of a drug. Hybrid parameters such as C_{max}/AUC may be a more robust index than C_{max} of the overall shape of the drug concentration vs. time curves. Additional objectives of the dosage proportionality study are an assessment of the variability of the pharmacokinetic parameters and a detailed evaluation of the elimination kinetics of the unchanged drug, all of which are useful for planning subsequent studies. This study also presents an opportunity for a critical assessment of the bioanalytical method prior to its use in pivotal bioavailability, bioequivalence, and dosage form proportionality studies. The drug is usually administered to a panel of healthy normal volunteers as a solution or a suspension, since, as with the ADME studies, intrinsic pharmacokinetic properties unobscured by dosage form factors are desirable.

In a 3 × 3 replicated Latin square design, 2.5, 5, and 10 mg solutions of PN 200-110 were administered to 15 normal healthy male volunteers. Successive administrations of drug

were separated by a 7-day washout period. Blood was

obtained at fixed time points between 0 and 12 h postdose and plasma concentrations of PN 200-110 determined by a specific radioimmunoassay (RIA). The RIA provided greater sensitivity than the HPLC method used previously (see Section III.C.1). The sensitivity advantage over HPLC frequently offered by RIA and other immunological binding assay methods can be offset by specificity problems. Therefore, the validity of the RIA to measure PN 200-110 selectively in plasma was demonstrated by extensive crossreactivity evaluations with isolated PN 200-110 metabolites and by comparison of the RIA data in clinical samples with an alternative analytical method of proven specificity, in this case gas chromatography coupled with mass spectrometry (GC/MS) [56]. The derived parameters C_{max} and t_{max} were obtained by inspection and AUC by the trapezoidal rule. Interpolated parameters and AUC (0-∞) were calculated from the best fit equations using a triexponential expression with a lag time to absorption for the curve-fitting process [63,64]. Descriptive statistics were calculated for derived and interpolated parameters and for their logarithmic transforms; the latter was done to minimize the influence of outlying values. Linear regression analysis was performed for the relationship of parameters with the dosage. The appropriateness of a straight line through the origin for this relationship was examined using a test for lack of fit [71]. Analysis of variance was performed on the parameters to assess the effects, if any, of study period and dosing sequence. Analysis of variance on dose-corrected parameters represents an alternative approach to test their relationship with the dose. From the error terms in the analysis of variance the number of subjects necessary to discern a given difference in parameter means with an adequate degree of statistical assurance (power calculations) was determined for application to future pivotal bioavailability studies.

As expected from the pilot and ADME studies previously discussed, the dosage proportionality study confirmed a linear dose-bioavailability relationship for PN 200-110. No sequence or period effects were noted. Intersubject coefficients of variation of about 40% for AUC, 50% for C_{max}, as well as a large error term in the analysis of variance suggested considerable within- and between-subject variability for PN 200-110 bioavailability parameters. The power calculations revealed that to discern 20% differences in parameter means at least 16 subjects would be required for AUC and at least 29 subjects for C_{max}. Bioavailability differences $\leq$20% have traditionally been considered therapeutically not relevant [72], and this concept still generally applies to the drug-drug interaction studies, or studies exploring the effect of age or disease state on drug bioavailability, discussed later in this chapter. As previously discussed, it does not apply to bioequivalence of drug products, in which the differences in mean AUC, C_{max}, or t_{max} usually must be considerably smaller than 20% to achieve declaration of equivalence by confidence band criteria [21]. Nevertheless, power calculations based on

20% (or other differences of interest) can provide useful

insight as to the minimum subject panel size required to achieve the desired degree of statistical assurance.

It is apparent from the dosage proportionality study that except for the quantification of parameter variability and a final comprehensive validation of the bioanalytical method, little additional information on the pharmacokinetics of PN 200-110 was obtained. However, particularly for drugs for which nonlinearity may be an issue as suggested from the results of the preclinical PK studies, the single dose clinical safety and toleration study, or from observation of nonlinear accumulation of drug in the multiple dose safety and toleration study, a crossover dosage proportionality study is highly desirable. In retrospect, it may have been possible to combine the PN 200-110 dosage proportionality study with the intrasubject variability study discussed below. In this combined study protocol involving four treatment periods, 2.5, 5, and 10 mg PN 200-110 as a solution and a repeat of one of the three dose strengths would be administered in randomized order, providing both dosage-bioavailability and more extensive within- and between-subject variability data.

3. Intrasubject Variability Study with PN 200-110

Phase I studies are normally conducted with simple dosage forms. They usually are more sophisticated formulations than the filled capsules used for animal toxicology studies or radioactive ADME studies, containing various excipients and demonstrating product stability for >3 months. However, the phase I dosage forms (e.g., clinical service form) often are rudimentary compared to the finished product for marketing, owing to technical difficulties and incomplete knowledge of required dosage strengths encountered this early in a drug's development. There is also little justification at this stage for the considerable expense of generating tablets, liquid capsules, or other acceptable market forms, since most investigational drugs do not advance beyond phase I or II. Pharmacokinetic data obtained in phases I and II may provide an indication of desirable drug input characteristics, which should be considered when devising the final dosage form and strengths. For all of these reasons, it is likely that the dosage form to be marketed will not be available until well into clinical phase III, with most or all of the clinical studies done with the clinical service form. The final dosage form can be introduced by demonstrating bioequivalence with the clinical service form, or by proving clinical equivalence between final form and service form. Proving clinical equivalence can be a difficult logistical problem because of the extent and timing of the final dosage forms' introduction into the clinical program, blinding issues, and choice of criteria for determining clinical equivalence. However, the more expeditious process of introducing the form via a bioequivalence study also carries risk, since a demonstration of equivalence can be

difficult for certain types of drugs or formulations. Thus, a crucial element of the bioavailability regulations has been that a bioequivalence study should have

sufficient power statistically to detect at least a 20% difference in the means of two treatments at a of 0.05 and b of 0.2. A power of 0.8 or better ensures the acceptability of the study statistically [17]. This means that proof of bioequivalence is based on average results for the rate and extent of absorption of reference and test product and appropriate measures of variability. The number of subjects in the study must be sufficiently large to ensure a high probability (>0.8) of detecting a 20% difference in mean bioavailability between reference and test product if such a difference does indeed exist. A difference in the means of <20% is not believed to be clinically important, as previously mentioned [16]. In addition to the requirement dealing with the average rate and extent of absorption, rules were developed concerning the relative bioavailability of drugs in individual subjects [18,72]. They relate to the range of allowable bioavailability within a given study, specifying that the relative bioavailability of the test product must be >75% for at least 75% of the subjects (75/75 rule) or >70% for at least 70% of the subjects in certain cases [73]. This early attempt to use the ratios of pharmacokinetic parameters for bioavailability assessment was abandoned in 1988 because of serious statistical flaws [19,74]. The numerous subsequent models and proposals for the analysis of individual bioavailability ratios and criteria for declaring bioequivalence have by consensus been condensed to acceptance ranges of 80125% for the 90% confidence interval for (log transformed) AUC ratios [21,75,76], with C_{max}, as well as C_{min} for steady-state studies, also judged by these criteria. Bioequivalence in t_{max} is not essential unless onset of action or safety are important clinical considerations. The statistical power and confidence bands criteria for a study are dependent upon the within- (intrasubject) and between-subject (intersubject) variability of bioavailability for the drug in question. Changes in the study design, such as increasing the number of subjects, alternative choice of reference product with more reproducible pharmacokinetic properties, or utilizing a steady-state rather than single dosage-design, have been proposed as a means of ensuring the statistical acceptability of a study [18]. However, drugs showing a large variability of pharmacokinetic parameters, possibly associated with a high first-pass effect, may not necessarily provide acceptable bioavailability studies even with these changes in study design [19,77].

The FDA BD Code [78] identifies those well-studied drugs known to have bioequivalence problems and for which statistically acceptable studies cannot be conducted. In vitro dissolution data may suffice to demonstrate bioequivalence for these drugs. In the case of a new drug, however, failure to provide a statistically acceptable study may not be ascribed to a bioequivalence problem without obtaining evidence that such a problem indeed exists. This would include demonstration of a very large first-pass effect, the existence of high within- and between-subject variability, or dissolution problems of the

drug substance [18], although it is not quite clear what degree of first-pass effect,

or variability, and what type of in vitro dissolution protocol would compel FDA to waive bioequivalence testing and accept new formulations based on comparable in vitro dissolution.

The bioavailable fraction of PN 200-110 determined in the single-dose ADME study was 18%. The dose-proportionality study revealed high intersubject coefficients of variation for AUC and C_{max}. The panel size projections from the dosage-proportionality study indicated that at least 29 subjects would be necessary to achieve statistical acceptability for all comparisons of the means pharmacokinetic parameters. Although all of these findings are suggestive of a bioequivalence problem with PN 200-110, a more definitive assessment of the intrasubject variability of the drug was needed, prompting the following study on the variability of PN 200-110 bioavailability parameters.

A panel of 13 normal healthy male volunteers received a single oral dose of 10 mg PN 200-110 as a solution on three separate occasions, separated by a 7 day washout period. Plasma levels of PN 200-110 were determined between 0 and 12 h postdose, and derived parameters were obtained as previously described. Analysis of variance was performed on the data, employing a randomized block design as well as nested-design analysis. Power calculations were also performed. Individual bioavailability ratios were determined, and the degree of correlation of individual parameters between the three treatment periods was assessed.

The average bioavailability data for PN 200-110 solution showed good repeatability. Differences between the three treatment periods for mean AUC, C_{max} and t_{max} were <10% despite considerable within- and between-subject variability. This variability is displayed graphically in Figure 7. From the mean square error and the confidence interval of the analysis of variance the within-subject coefficients of variation were calculated to be 20%, 21%, and 28% for AUC, C_{max} and t_{max}, respectively, with the 95% confidence intervals ranging from about 15 to 30% for AUC and C_{max} and 20 to 36% for t_{max}. Further evidence for high variability was the lack of statistical significance for the intrasubject correlation analysis. It was concluded from this study that the variability inherent in PN 200-110 disposition may on occasion render the bioavailability comparison of a reference and a test formulation of this drug statistically unacceptable, even if the two formulations are indeed identical and a crossover design with as many as 30 subjects is utilized. However, bioavailability assessment of PN 200-110 dosage forms should provide an accurate reflection of their true average rate and extent of absorption.

Given the uncertainty of proving bioequivalence between the clinical service form and a new market form development, it was decided to develop a clinical service form of PN 200-110 that would also be suitable for marketing.

The confidence band approach, being based on sounder statistical principles than the 75/75 rule, represents an important step forward in bioequivalence testing. However, its use by regulators as an all-or-nothing decision criterion of the

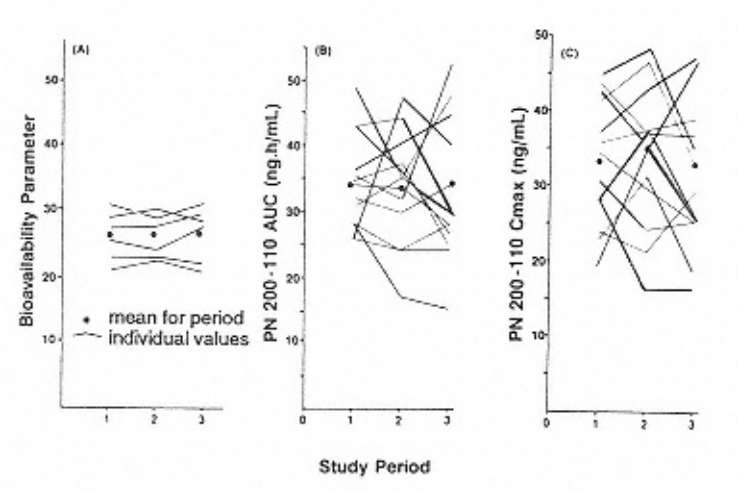

Figure 7
Individual AUC values for a hypothetical drug possessing low inter- and intrasubject variability administered to a panel of six subjects on three separate occasions (A). Individual AUC (B) and C_{max} (C) values in normal subjects administered a 10 mg solution of PN 200-110 on three separate occasions.

acceptability of a test vs. reference product prompts the need in early drug development to devise strategies that avoid or reduce the risk of failing this crucial test. Thus, the intrasubject variability study should be scheduled sufficiently early in drug development to allow a timely decision for the manufacture of, and obtaining adequate shelf-life data for, a dosage form that either can be expected to pass bioequivalence testing or for which adequate clinical experience must be obtained.

4. Absolute Bioavailability Study with PN 200-110

Upon completion of the clinical phase I and early phase II pharmacokinetic studies described above, sufficient pharmacokinetic and bioavailability data should be available to optimize the extended phase III clinical trials, as well as the pharmacokinetic studies performed at that stage of drug development. For certain drugs presenting a bioavailability problem, the conduct during late phase I or early phase II of an absolute bioavailability study, comparing drug concentrations and pharmacokinetic parameters after oral and parenteral administration of drug, may be informative. Additional toxicity studies in animals may first have to be performed to demonstrate the safety of the parenteral route for

humans. An amendment to the original IND containing appropriate manufacturing, biopharmaceutical, and animal safety data may be filed for this purpose. If both the oral and parenteral routes of administration are proposed for therapeutic use, their development usually proceeds under separately filed, although suitably cross-referenced, INDs.

The technique of quantitative mass spectroscopy of stable isotope-labeled drug has been shown to be useful for absolute bioavailability and other pharmacokinetic studies [79]. Coadministration of the nonlabeled drug with the stable isotope-enriched analog(s) as internal standard(s), followed by mass spectroscopic determination of these compounds, provides individual drug level profiles and relative bioavailability data from a single set of the biological sample. The advantages of this approach are therefore a possible consolidation of several administration periods into one and, by reducing the variability inherent in multiple administrations, a smaller subject panel size necessary for statistical acceptability. This technique is particularly useful in studying drugs showing high intrasubject variability. These advantages are offset by the requirement for chemical synthesis of the isotope-labeled standard(s) and the necessary absence of changes in pharmacokinetic and biotransformation processes due to the presence of the stable isotope label, which may have to be verified in an independent study. Another requirement is the presence of linear pharmacokinetics under the dosage and conditions of the stable isotope study. In a typical trial, the stable isotope internal standard is administered orally (for bioavailability assessment) or intravenously (determination of absolute bioavailability) followed immediately by an oral dose of the nonlabeled drug formulation to as few as four to six subjects. Various permutations of study design and frequency and sequence of administration of nonlabeled and isotope enriched drug are possible as discussed by Wolen [80]. An example of the use of stable isotope-labeled PN 200-110 in a pharmacokinetic study establishing the drug's absolute bioavailability in normal subjects and subjects with hepatic impairment is provided in Section VI.B.3.

The use of radiolabeled drug in an absolute bioavailability study should be considered for drugs that are subject to poor and/or erratic absorption, high first pass effect, or in which changes in biotransformation or other processes are suspected as a function of route of administration. To be considered are study designs including a third intravenous cohort in the earlier discussed ADME study (see Section III.C), or a separate crossover oral intravenous (i.v.) trial, provided that the protocols involving multiple administrations of radiolabeled drug are justifiable based on radioactivity exposure considerations (see Section III.C.2). Assessment of the chemical and radiochemical stability of the drug solution is mandatory.

B. Pharmacokinetic Support of Phase II Clinical Trials: Population Pharmacokinetic Screen with ZK 112-119

The pharmacokinetic studies and the dosage escalation trials in normal volunteers conducted during clinical phase I should provide maximum safe average drug concentrations and dosage. The concentration profiles obtained in these studies should assist in defining a dosing frequency that does not result in excessive drug accumulation. This, as well as an appreciation of the drug's pharmacokinetic variability and biopharmaceutic properties, should assist in the design of the clinical phase II trials. The phase II trials evaluate the drug's therapeutic potential in patients and include an assessment of chronic safety in a larger population than used in phase I. Direct pharmacokinetic support of the phase II clinical trials may involve individual optimization of drug dosage based on monitoring drug concentrations in patients [81], conduct of concentration-controlled titration studies [82], or an assessment of population pharmacokinetics, preferably in conjunction with pharmacodynamics [2227]. Monitoring drug concentrations without linkage to therapeutically relevant effects or their surrogate measures is of limited usefulness except when pharmacokinetic variability is high, compliance is poor, or pharmacodynamic variability is negligible [83].

Clinical trials involving randomized concentration control represent a prospective approach to linking drug dosage, concentration, and effect. In contrast, the population kinetics/dynamics evaluation of a clinical trial represents a retrospective analysis of important demographic, physiological, and pathological correlates that may be used in turn to predict an individual's response to the drug. Either one or both approaches can be applied to the phase II (and phase III) clinical trials. Although the randomized concentration-controlled clinical trial (RCCT) involving a parallel or titration design represents an attractive concept for phase II investigations [84], there are as yet few reports of its successful application at this stage of drug development. A discussion of an RCCT conducted during clinical phase III is contained in Section V.E. The population pharmacokinetics concept, after having received considerable attention to its theoretical and computational aspects [2225], is now well-represented in the literature with practical examples [8587]. Such an example, involving the drug ZK 112-119 (see Fig. 1), is also provided below. The majority of population pharmacokinetic evaluations are model-based using NONMEM analysis, although model-independent approaches have also provided useful insights into the relationship of plasma concentrations with patient demographics and measures of safety and efficacy. Both approaches, a model-independent quartile classification method and NONMEM analysis using a cubic-linear spine model, were used to analyze the ZK 112-119 plasma concentrations collected during the phase II clinical investigation of this anxiolytic agent. The safety and efficacy of ZK

112-119 was evaluated against placebo and an active control, a marketed

antianxiety agent, in two separate but simultaneous multicenter (8 centers each) studies involving 371 patients with generalized anxiety disorder. Each study used a randomized, double-blind, parallel group design. Following a 1 week drug-free baseline screening period, patients were randomized to receive ZK 112119 (67 in study 1, 58 in study 2), active control, or placebo for 6 weeks, with the dosage titrated from 1 mg (study 1) or 2.5 mg (study 2) ZK 112119 capsules three times daily to a maximum of 3 mg three times daily (t.i.d.) (study 1) or 7.5 mg t.i.d. (study 2) by study week 4, followed by a 2 week double-blind medication reduction period. A single blood sample for plasma ZK 112119 measurement at the end of study weeks 2 (1 week on medication), 3, and 4 (just prior to dosage de-escalation period) was obtained. The blood draw took place during the scheduled clinic visit of the patient; time of day was not controlled. Plasma was harvested, tubes appropriately labeled, and a case report form specifically generated for each sample was completed with the following information: study number, center, patient identification, study week, date, and exact time of blood draw. The form also recorded information elicited from the patient, including date and time of last dose, dosage regimen during the last 72 h, and concomitant medications during the last 72 h. Dietary history was not investigated because of the absence of a food effect for the ZK 112119 capsules. The plasma samples accompanied by their case report forms were shipped from the clinical sites to the analytical laboratory either individually at ambient temperature in cardboard mailers provided to the clinical investigators for next-day delivery, or were frozen in bulk, depending upon individual center preference. Upon receipt at the analytical laboratory, all samples were stored frozen until analysis. ZK 112119 in spiked and clinical samples was found to be stable under the shipping, storage, and handling conditions. Of the 125 ZK 112119 patients entered, 92 completed the study and 86 (51 in study 1 and 35 in study 2) provided valid plasma samples for a total of 220 ZK 112119 plasma concentration data points used for statistical analysis. Thus only about 60% of the 86 completers provided the set of three plasma samples per patient specified by the protocol. This points to the considerable logistical difficulties and resource requirements for the conduct of a population pharmacokinetic trial. Of the total 890 plasma samples collected during the study, 38 had to be discarded because of inadequate identification, reaffirming the importance also of attending to small details to ensure success of a clinical pharmacokinetic or population pharmacokinetics study. Without the opportunity to break the study code, a resource investment of 852 analyses was required to yield the 220 data points for the active, ZK 112119 group. This 220 point data set was equally spread across the three collection periods in study weeks 2, 3, and 4, and also well balanced across ZK 112119 dosages, with the 2, 2.5, 3, and 7.5 mg doses representing approximately 85% of the data points. Nearly 90% of the data set was collected during the period 27 h after the morning dose, with the data spread relatively

evenly over this time interval. A reasonably balanced data set strengthens the study conclusions.

For the model-independent analysis, the ZK 112119 08 h concentration data were dose-normalized, pooled by 1 h time intervals, and each data pool classified into concentration quartiles. The boundaries for these quartiles are shown in Figure 8 as are the associated dose-normalized areas under the quartiles vs. time curves (AUQ) and the clearances, which were calculated from the ratio f/AUQ, with f as the fraction of ZK 112119 bioavailable [62] held constant. In studies with young normal male subjects, plasma clearances of ZK 112119 ranged from 19 to 43 L/h, which is similar to the calculated clearances for patients in quartiles 1, 2 and 3. Female patients were significantly more prevalent in the higher quartiles, males in the lower quartiles. The six Afro-American patients in the study were distributed in the higher quartiles, Q3 (n = 3) and Q4 (n = 3). No other demographic variables, such as patients' weight or age, or any safety variables, showed significant associations with the concentration data. However, there was a significant positive correlation of the AUQ with the frequency and degree of sedation experienced by the patients.

From the normal volunteer pharmacokinetic studies with ZK 112119, an empirical spline model was derived involving a log-cubic plasma concentration decline up to 6 h postdose, and after 6 h postdose a log-linear plasma concentration decline. The model was applied to the patients' data using NONMEM analysis to identify dependencies of model parameters on demographic and clin-

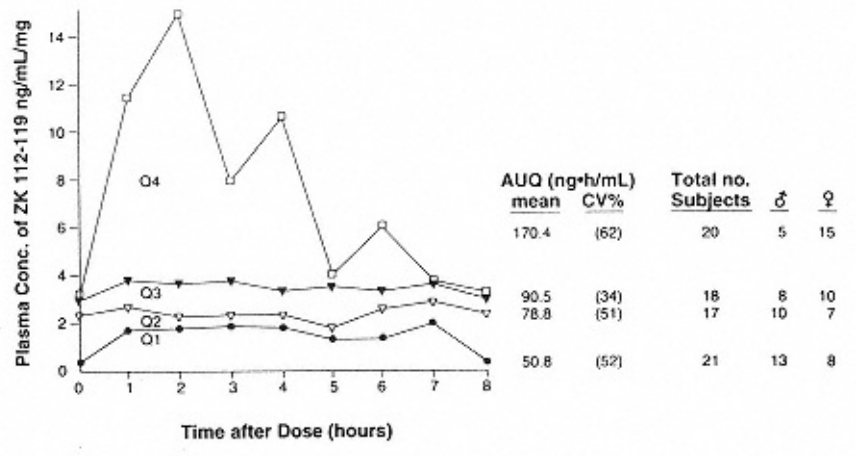

Figure 8
ZK 112119 concentration subgroup profiles for 86 patients completing a population kinetic analysis of a 4 week phase II safety, toleration, and efficacy trial.

ical variables. Using Bayesian methods, an AUC was estimated for each patient and study week. The AUCs were strongly correlated with the dosage and with the AUQs. The correlation of the model parameters with the demographic variables age and race was statistically significant, demonstrating higher concentrations and slower drug elimination rates for patients >40 years of age irrespective of gender and race, and for the African-American patients irrespective of gender and age. This parallels the results of the model-independent analysis. Also, the observation by the model-independent approach of a strong, positive correlation between the frequency or degree of sedation and the AUQ was paralleled by the model-dependent data showing a strong positive correlation of sedation with plasma concentration, and a positive, although weaker, correlation with the Bayesian AUCs. There was no apparent correlation for any of these measures, using model dependent or independent approaches, with the Hamilton anxiety scale, an index of the efficacy of ZK 112119 to reduce the symptoms and/or manifestations of anxiety.

The population pharmacokinetic screen of the two double-blind positive and negatively controlled safety and efficacy studies of ZK 112119 yielded useful information on the disposition of this experimental anxiolytic agent in patients. The study provided confirmation in patients of dosage proportionality previously established in healthy male subjects. It also demonstrated an effect of age, gender, and race on the drug concentrations achieved with a given dosage. The study showed a positive relationship of dose or plasma concentration, with sedation, a side effect of the drug. There was no such relationship with efficacy based on an assessment of patient status using the standardized Hamilton scale. This information was extremely valuable in planning the phase II trials with this compound. Thus, although very resource- and labor-intensive, phase II population pharmacokinetic screening should be considered for drug development. Early breaking of the study code, if feasible, would eliminate the need for drug analysis in nonrelevant samples. However, the savings in resources must be weighed against information on possible misdosing and noncompliance gained by analyzing both placebo and active control samples for the experimental drug.

V. Clinical Phase III

A. Pharmacokinetic Considerations in Developing the NDA

The scope of pharmacokinetic studies performed in clinical phase III represents not only a continuation and elaboration of previous work on drug biotransformation or dosage form performance but also, if not already started in phase II, the initiation and conduct of a new group of studies to determine the effects of such variables as different patient populations, disease states, or environmental conditions on drug disposition. These studies are also referred to as labeling

studies because they provide information for the appropriate use of the drug in patients as eventually presented in the package insert. By specifically focusing on a single variable, labeling studies can complement and enhance the labeling information for general use of the drug projected from the clinical data of phase III, whether the clinical trials were performed in hundreds or thousands of patients. However, the number of and the protocols for labeling studies usually vary according to drug type, properties, and use. Pharmacokinetic studies of dosage form performance of clinical service forms vs. forms proposed for marketing must be completed in phase III, and investigations of possible advantages imparted by the use of novel dosage forms, such as oral controlled-release dosage forms, skin patches, implants, or pumps, should be seriously considered at this stage. However, registration activities for these novel dosage forms most often occur under a separate IND and at a time when the drug, contained in a standard dosage form, is already an approved entity.

The extent and complexity of investigations on the biotransformation of the new drug depend upon drug class and use, although in general phase III activities greatly exceed those performed in phase II. This usually is not a consequence of planning but results from the time-consuming difficulties inherent in isolating and identifying multiple metabolites that are usually present in minute quantities and as complex mixtures in large volumes of biological fluid. A major program to synthesize the principal metabolites chemically must frequently be undertaken because insufficient quantities of metabolic products are available from the ADME studies. This is especially critical in the situation in which biotransformation in humans proceeds to different products than in the animal species used for toxicity testing. The only alternative is to undertake additional safety assessment of the synthetic human metabolites in animals. An approach that is often utilized is to synthesize potential metabolites early in the drug development process for pharmacological and safety assessment. Examples of likely candidates for synthesis are products of N-demethylation, de-esterification, and hydroxylation of labile sites. Computer programs have been devised to assist in evaluating relevant routes of biotransformation of any chemical structure [88]. All these considerations had an impact on the biotransformation work performed for PN 200-110 in clinical phase III, which included the following:

Detailed elaboration of the major metabolic pathways in humans and all species involved in toxicity testing: These pathways included de-esterification, oxidative processes leading to aromatization, and glucuronidation and other conjugation reactions. Humans and other animals shared similar routes of bio-transformation for PN 200-110, although some quantitative differences were noted [89].

Chemical synthesis and evaluation of pharmacological activity of metabolites and potential metabolites of PN 200-110: None of these compounds showed appreciable activity [90].

Investigation of the role of metabolites in the accumulation or retention of radioactivity in the human ADME studies and their presence or absence in the toxicity species: If certain PN 200-110 metabolites had been found to be pharmacologically active, development and validation of bioanalytical methodology for these metabolites and studies on the dynamics of their generation and elimination would be desirable. This would have had an impact on the conduct of phase III bioavailability and bioequivalence studies (see Table 2). Because of the absence of active metabolites, these studies were analyzed for parent PN 200-110 only.

PN 200-110 is a racemate with the principal pharmacological activity of calcium channel blockage residing in one of the enantiomers [91]. There has been considerable polarization of opinion regarding the development of a racemate drug vs. its active enantiomer and the necessity for using stereoselective assays in pharmacokinetic studies for this type of drug [92]. The mainstream consensus accepts development of a racemic drug and use of nonstereoselective bioanalytical methodology, although it would be desirable for the method's validation to include evidence for the constancy of enantiomer ratios throughout a dosing interval and over the therapeutic dosage range [44,93]. A determination of PN 200-110 plasma concentrations in the dosage proportionality study (see Section IV.A.2) by achiral gas chromatography/mass spectrometry [56] and chiral radioimmunoassay [57] showed constant ratios, indicating the absence of chiral effects in the drug's disposition.

Knowledge of metabolite activity at the earliest possible opportunity facilitates planning of the entire drug development program. The specific consequences for phase III pharmacokinetic studies resulting from the presence of active metabolites are determined by the precursor or successor relationship of active and inactive species, the time course of conversion, and the relative amounts converted. One extreme is represented by an inactive prodrug that is rapidly converted in vivo to a single pharmacologically active species; the other extreme is the formation of a series of metabolites, each presenting a different spectrum of activity, from an active parent drug. In the former case measurement of the single active compound should suffice to address the requirements posed by scientific and/or regulatory considerations. For the latter, individual monitoring of all, or at least the major, active species may be required, since the basis for demonstrating bioavailability according to 21 CFR 320.23 and 320.24 [8] is the measurement of the active drug ingredient or therapeutic moiety or its metabolites in biological fluids as a function of time. FDA personnel have interpreted that to mean the following: where there is rapid first pass metabolism, where the metabolite is either present to a much larger extent or has significantly greater pharmacological action the Division of Biopharmaceutics will require that the active metabolites generated also be

measured in addition to the parent

drug [94]. It can be assumed that the regulatory requirement for appropriate statistical assurance of detecting parameter differences not attributable to subject variability (21 CFR 320.23) holds for metabolite concentration determinations as it does for parent drug.

B. Regulation Bioavailability and Bioequivalence Studies

1. Dosage Form Proportionality

In accordance with FDA guidelines [8], the regulation bioavailability study for the PN 200-110 capsules to be marketed, a formulation identical in size and composition to the clinical service capsule, was performed in comparison to a reference material, a solution of the drug. The protocol for this study also incorporated relevant findings from the intrasubject variability study described previously, in order to increase the likelihood of achieving adequate statistical sensitivity or power to detect clinically significant differences in the rate and extent of PN 200-110 absorption not attributable to subject variability. An objective in addition to determining the relative bioavailability of the capsule vs. the solution reference standard was to establish the bioequivalence of equipotent drug treatments of all capsule strengths to be marketed (2.5, 5, 7.5, and 10 mg). The capsules, containing identical ingredients and showing similar in vitro dissolution profiles, differed in the ratio of active to inactive ingredients. The PN 200-110/excipients ratios ranged from 1:107 for the 2.5 mg capsule to 1:26 for the 10 mg capsule, with the 5 and 7.5 mg capsules showing ratios within this range. Consequently, if PN 200-110 treatments of 4 × 2.5 mg capsules and 1 × 10 mg capsules were shown to be bioequivalent, this would also imply equivalence between equal dosages of either of these two capsule strengths and the intermediate 5 and 7.5 mg strengths (i.e., dosage form proportionality). Of the study's two objectives (to define the bioavailability of the PN 200-110 capsules relative to the solution, and to establish bioequivalence between the two capsule strengths), the latter requires a statistical approach based on confidence bands as discussed for the 2 × 2 crossover study discussed in Section III.C.3. The former objective of relative bioavailability can be supported with the statistical power concept. Combining the statistical power and confidence band approaches in a single study is appropriate provided that the interpretation of the data is guided by the specific limitations inherent in these approaches, as becomes clear from the results of the PN 200-110 dosage form proportionality study.

In a replicated 3 × 3 Latin square design, a panel of 24 normal healthy male volunteers received single 10 mg oral doses of PN 200-110 as a 10 mg capsule, as 4 × 2.5 capsules, or as a 10 mg solution following an overnight fast. A washout period of 7 days separated each drug treatment. Plasma concentrations of PN 200-110 were determined at fixed time points between 0 and 24 h postdose. The mean plasma level data for the 24 subjects are

shown in Figure 9.

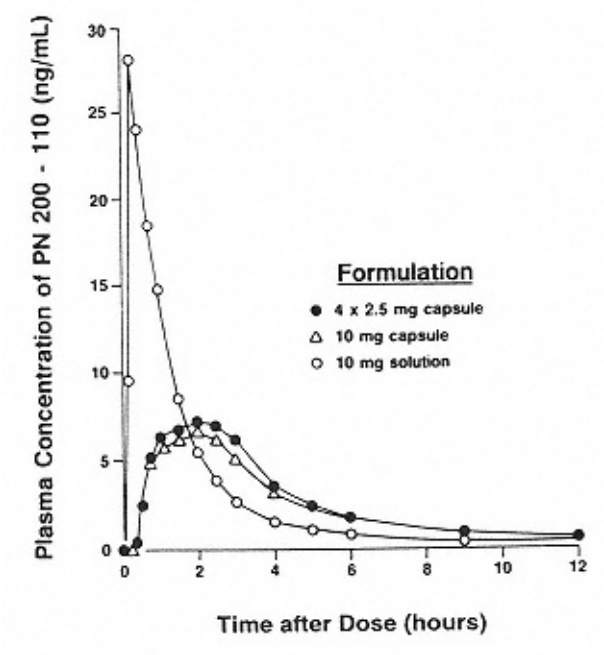

Figure 9
Mean plasma levels of PN 200-110 in normal volunteers administered single oral doses of drug as a 1 × 10 mg capsule, 4 × 2.5 mg capsules, and a reference solution in a crossover design.

Bioavailability comparisons using methods as already described for the bioequivalence study with ZK 112119 (see Section III.C.3) demonstrated a significantly slower rate of absorption, and a substantially lower mean C_{max} for the capsule, by 72% compared to the solution and a slightly though also statistically significantly reduced mean AUC, by 15% compared to the solution. These results are similar to those obtained in the comparison of the early capsule formulation with the solution in the pilot pharmacokinetic study (see Section III.C.1), demonstrating the utility of pilot studies for dosage form development.

The intrasubject variability (calculated from the mean square error of the analysis of variance and expressed as CV%) for AUC, C_{max}, and t_{max} of the solution, as well as the AUC values of the two capsule formulations, ranged from 20 to 28%. This is in excellent agreement with the variability observed for the solution in the intrasubject variability study. In contrast, the intrasubject variability for C_{max} and t_{max} of the capsule treatments was considerably higher and ranged from 38 to 49%, approaching the intersubject coefficients of variation

(CV%) for the parameter means. The rate of absorption of a solid dosage form frequently is more variable than that of a solution (the ideal formulation), demonstrating the need to weigh carefully the utility of using a solution vs. a solid dosage form in the intrasubject variability study (see Section IV.A.3). Pharmacokinetic variability assessment with the clinical service capsule adds to an understanding of the capsule's performance in the clinical program. This should be weighed against the lesser relevance of such a study when conducted with a prototype service form that may experience significant changes prior to its ultimate use in pivotal clinical studies.

In parallel with the nearly fourfold higher C_{max} of the solution than the capsule (see Fig. 9), the variance for the solution was >10-fold higher, a statistically significant difference by the Pitman-Morgan test [67]. The 4 × 2.5 mg vs. 1 × 10 mg capsule comparison therefore employed analysis of variance for a randomized block design and excluded the solution data. For the capsule comparison, the two one-sided t-tests procedure as proposed by Schuirmann [68], Westlake's symmetrical 95% confidence intervals [69], and the conventional 90% confidence intervals [21] all corroborated bioequivalence for AUC (024 h). Differences in mean C_{max} and t_{max} between the 4 × 2.5 mg and 1 × 10 mg capsule treatments were <8%, but the low statistical power, lack of significance of the one-sided t-tests, and confidence intervals slightly larger than acceptable reflected failure to achieve a rigorous demonstration of bioequivalence for C_{max} or t_{max}. Curve fitting of the plasma concentrations using a triexponential expression including lag-time to absorption [64] yielded excellent fits of the observed data with the calculated curves as based on regression coefficients, or, even better, visual inspection. Using the calculated parameters (AUC $0\text{-}\infty$, interpolated C_{max} and t_{max}) for the bioavailability comparisons resulted in no (for AUC) or only marginal (for C_{max} and t_{max}) changes to the bioavailability conclusions. The similar results for the raw (non-curve-fitted) and curve-fitted data are consistent with the regulatory emphasis on the raw, rather than curve-fit, data for bioavailability comparisons [21]. Furthermore, sufficiently frequent blood sampling to define C_{max}, t_{max}, and the elimination phase (for calculating residual AUCs) adequately should obviate the advantage offered by curve-fitting of yielding interpolated C_{max} and t_{max}, and AUC$(0\text{-}\infty)$. However, curve-fitting can be useful in attenuating the influence of outlier concentrations.

As was anticipated by the intrasubject variability study, bioequivalence by strict criteria could not be uniformly declared, since the 90% confidence intervals for the ratio means of the log-transformed parameters for the capsule treatments were slightly outside the mandated 0.81.25 window for C_{max} (although slightly within the window for curve-fitted C_{max}). The 90% confidence interval appears to be the single most relevant bioequivalence measure in regulatory thinking [21]. The marginal results obtained for the regulation

bioavailability study

of the PN 200-110 capsules justifies the decision to gain clinical experience with the capsule dosage form and strengths intended for marketing, instead of solely relying on a bioequivalence study of uncertain outcome to switch from the clinical service form to another form for the market.

2. Bioequivalence Study (Steady-State)

The design frequently encountered in a regulation bioavailability study, that of comparing the dosage form to be marketed with the clinical service form using a solution of the drug as reference standard, was also applied in a study comparing the capsule form used in clinical trials of the drug HC 20511 (ketotifen; see Fig. 1 for structure) with the tablet proposed for marketing. Instead of the usual single-dose administration, this study was performed at steady state, that is, making the appropriate parameter comparisons after each dosage form (ketotifen capsule, tablet, and solution) was administered for a sufficiently long period to achieve the steady-state concentrations. Linear pharmacokinetic theory indicates that the area under the curve for a dosing interval equals the AUC (0-∞) after a single dose of the drug [64] and provides the rationale for a steady-state study. Because of the greater complexity and cost of a steady-state study and the underlying principle that subjects should not be unnecessarily exposed to a drug, whether experimental or approved, this design should be reserved for those instances in which a single-dose bioavailability or bioequivalence study would be unsuitable. Based on bioavailability regulations [8] steady-state studies should be considered when:

There is a difference in the rate of absorption but not the extent of absorption.

There is excessive variability in bioavailability from subject to subject.

The concentration of the active drug ingredient resulting from a single dose is

too low for accurate determination by the analytical method.

The steady-state design for the bioequivalence study of HC 20511 was chosen primarily because the bioanalytical method was not sufficiently sensitive to determine drug levels after a single 2 mg dose over three half-lives (the mean $t_{1/2}$ of HC 20511 in normal subjects is about 23 h). A five half-lives rule was initially prescribed by the bioavailability regulations [8]. A more realistic and commonly accepted approach is to measure drug concentrations accurately and precisely to three half-lives although even this could be considered overly restrictive in those instances in which the terminal half-life of a drug does not appreciably contribute to its AUC. An alternative approach that should be acceptable to the regulatory agency is to measure drug concentrations to 1/10 of peak above the limit of quantitation of the bioanalytical method [4345]. The alternative of

increasing the dosage of the single-dose administration to achieve measurable drug levels was not feasible for HC 20511 owing to possible subject intolerance.

In a three-period randomized crossover design, 21 healthy normal male volunteers received 2 mg doses of HC 20511 as a tablet, a capsule, and a solution. Subjects received one of the three dosage forms every 12 h for 7 days, immediately followed by the second dosage form every 12 h for 7 days and then the third dosage form every 12 h for 7 days. Thus, instead of the usual drug-free washout period in single-dose crossover studies, the present design simultaneously allowed washout of the prior dosage form and achievement of steady state with the next form to be evaluated. Blood samples were collected immediately prior to and for up to 12 h subsequent to dose 13 (day 7) and dose 27 (day 14) and immediately prior to and for up to 96 h subsequent to the final dose (day 21). Concentrations of HC 20511 were determined by radioimmunoassay. Figure 10 shows the mean plasma level data obtained during the three evaluation periods on study days 7, 14, and 2124. Achievement of steady state by days, 7, 14, and 21 was verified by similar 0 and 12 h C_{min} values and evaluating ketotifen half-lives during the washout period.

With the exception of one subject (who was excluded from the bioavailability comparisons), the terminal half-lives were <36 h. This implies that steady-state conditions were established for each formulation by dose days 7, 14, and 21, since 5 half-lives (corresponding to achieving >95% of steady state) represent

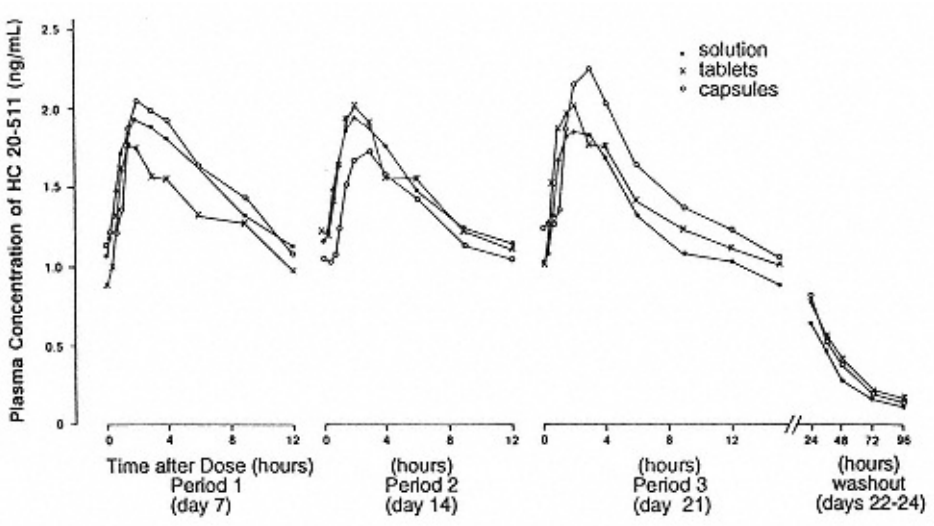

Figure 10
Mean plasma levels of HC 20511 determined on days 7, 14, and 21 in normal volunteers receiving 2 mg twice a day of ketotifen capsules, tablets, and reference solution over a 21 day period (3 × 7 day periods with crossover on days 8 and 15).

<180 h or <7.5 days. Bioavailability comparisons were based on evaluating the derived pharmacokinetic parameters (AUC, C_{max}, t_{max}, C_{min}), either observed or interpolated, using a three-exponential expression for steady-state levels [64]. Concentrations of HC 20511 from all three 12 dosing intervals plus the day 2124 washout period were curve-fit simultaneously. Elimination parameters were fit independently of formulation; however, best fits were sought separately for each of the three formulations with respect to input rates and the fraction of drug bioavailable compared to the reference solution. Statistical analysis of these parameters indicated bioequivalence of the tablet and capsule formulations. The analysis also showed acceptable bioavailability of the capsule and tablet compared to the solution reference standard, and demonstrated adequate statistical power to discern 20% differences, had they existed between the three formulations. All parameters except t_{max} met the 90% confidence limits criteria. FDA regulations specify that a drug product that differs from the reference material in its rate of absorption but not in its extent of absorption may be considered bioavailable if the difference in the rate of absorption is not detrimental to the safety and effectiveness of the drug product (21 CFR 320.23) [8]. For drugs that achieve their therapeutic effect upon long-term administration and for which the incidence of acute adverse effects is similar, irrespective of different rates of absorption between dosage forms, variability in the t_{max} should be of no consequence.

The clinical service forms and dosage forms to be marketed for PN 200-110 and HC 20511 showed extent of bioavailability (AUC) similar to that of the reference solutions. However, for some drugs, poor aqueous solubility, pK_a, and other physicochemical properties can adversely affect dissolution and result in loss of bioavailability. This occurs particularly for those drugs showing a high first-pass effect in which bioavailability depends upon the absorption flux. Further consequences may be highly variable and unpredictable bioavailability, compounded by environmental factors, such as food intake. These are the reasons that phenytoin was identified as a drug with high risk potential for bioavailability failures [95]. An evaluation of the bioavailability of various commercial dosage forms of phenytoin revealed considerable variability between them [96]. The challenge to the pharmaceutical formulator and manufacturer is to produce dosage forms with adequate and consistent bioavailability. Efforts to this end must be initiated very early in an NCE's development program, particularly for problem compounds. The conduct of clinical trials with poor dosage forms is clearly unacceptable. Despite their shortcomings as discussed in Sections III.C.1 and III.C.3, small pilot pharmacokinetic studies, typically involving as few as four to nine normal volunteers and a crossover design, are efficient means to evaluate dosage forms of drugs with high risk potential for bioavailability failures, unless a valid animal model has been elaborated. In vitro

dissolution

data can serve to prescreen dosage form candidates but usually are no substitute for in vivo evaluations.

C. Special Pharmacokinetic Studies (Labeling Studies).

In addition to the regulation bioavailability and bioequivalency studies performed in clinical phase III, special studies are often undertaken to answer specific questions that arise during the development process about the disposition of the compound. There are no federal regulations concerning which special studies are required for an NDA. However, specific observations of severe adverse reactions in some elderly subjects treated with nonsteroidal anti-inflammatory agents [97] focused on the drug dispositional differences in this population [98], and prompted interest in population pharmacokinetic studies to identify specific subpopulations, including the elderly, who may respond differently to the NCE and for whom special studies should be undertaken [99]. Thus, special studies are now normally conducted in selected segments of the population to determine the effects of such variables as demographics (age, race, and gender), disease state, or environmental factors (drug interactions and food) on the pharmacokinetics of the drug. This information allows a sponsor (manufacturer) to support dosage labeling claims (i.e., labeling studies) with definitive bioavailability and pharmacokinetic data. The following examples of special studies done with PN 200-110 are representative of those most commonly conducted. Depending on the specific indication of the drug as well as the intended patient population, additional studies may also be appropriate. On occasion, labeling studies should be performed in clinical phase II. For example, with a poorly soluble drug showing incomplete and/or erratic absorption in early pharmacokinetic trials, the effect of food on bioavailability may be considerable and the degree of food interaction should be known to optimize the conduct of the phase III trials. Conversely, not all of the studies specified may be required for all drug candidates, and, in a few cases, none may be necessary.

1. Effect of Age on PN 200-110 Pharmacokinetics

Because of the therapeutic indication for PN 200-110 (hypertension), it was likely that the target population would consist of a high percentage of elderly patients. Since numerous physiological changes occur in the elderly that can be relevant to drug disposition [98,100], this type of study is essentially mandated for any new chemical entity undergoing drug development.

In a 2 × 2 replicated Latin square design, PN 200-110 capsules (5 and 10 mg) were administered as single doses to a group of 26 healthy elderly (mean age 70, range 6583 years) male volunteers. Based on potential difficulties in recruiting an adequate number of

subjects at a single clinical site, the study was performed by two investigators with 13 subjects enrolled at each center. Administration of the two dose levels of the drug was separated by a 5 day washout

period. Plasma levels of PN 200-110 were determined at fixed time points between 0 and 24 h postdose. Bioavailability evaluations were based on the various derived parameters as previously discussed. There were no statistically significant differences between the bioavailability data from either study site. The mean plasma level data for the 26 elderly subjects following the 10 mg capsule dose in comparison to the mean plasma levels obtained in pharmacokinetic studies with young healthy volunteers (see below) are shown in Figure 11. Overall, for the 26 elderly subjects, both AUC and C_{max} exhibited a linear relationship with dose in the 010 mg range, as had been shown earlier for young normal individuals.

The mean bioavailability data for the 10 mg capsule in the elderly were compared with a database (n = 57 subjects) gathered from three previous PN 200-110 pharmacokinetic studies in which the same 10 mg capsule formulation was administered to young healthy male volunteers (mean age 28 years, range 1844 years). On average, the elderly showed statistically significantly higher AUC (by 55%) and C_{max} (by 25%), no change in t_{max}, and statistically significant reductions in the terminal elimination rate (by 40%), clearance (by 9%), and first-pass metabolism as determined by the fraction PN 200-110 bioavailable (by 41%). Individual AUC data for the young and elderly subjects are also summarized in Figure 11.

The bioavailability changes observed in the elderly in this study compared with young subjects are consistent with reduced drug elimination capacity or lower volume of distribution generally seen in the elderly population. Since PN

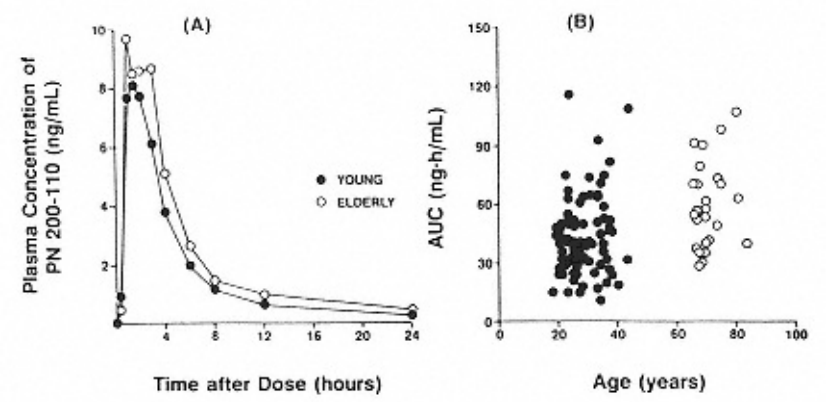

Figure 11
(A) Mean plasma levels of PN 200-110 in young (n = 57) and elderly (n = 26) normal subjects following administration of 10 mg capsules. (B) Individual AUC values obtained in young and elderly normal subjects.

200-110, as are other dihydropyridines, is subject to considerable intrasubject variability in the young and in the elderly, it would be necessary to apply this finding cautiously in any labeling (package insert) statements. A rational approach is to avoid a blanket proposal of dosage reduction in the elderly and instead suggest individualized therapeutic regimens of initially low dosages with subsequent titration to a safe and efficacious dosage level.

2. Effect of Renal Insufficiency on PN 200-110 Pharmacokinetics

Certain disease states can alter the pharmacokinetic and pharmacodynamic profile of a drug to such extent that dosage adjustments may become necessary. Of special importance are renal and hepatic dysfunction, since they can play a major role in altering drug elimination. Studies on the effect of disease state become almost mandatory if it has been shown previously that disease appreciably alters the pharmacokinetics of drugs that are structurally similar to the agent under development. Thus, it was observed that the pharmacokinetics of the dihydropyridines nifedipine [101] and nitrendipine [102] are variably altered when these drugs are administered to patients with renal impairment. Approximately 60% of the administered PN 200-110 dose is eliminated via the urine, almost exclusively in the form of metabolites (see Sections III.C.2 and IV.A.1), parallelling the results for other dihydropyridines [103]. These observations prompted conduct of a study of PN 200-110 in renal impairment.

Kidney function can be acutely or chronically impaired by intrarenal pathological processes affecting blood vessels, glomeruli, tubules, or the interstitium separating individual nephrons, or by extrarenal processes affecting kidney perfusion or urine flow. The overall effect on drug disposition may be reduced renal clearance, reduced renal metabolism, or both. However, since the kidney's metabolizing activity is roughly 15% that of the liver, the disease processes affecting the kidney by reducing excretory and metabolizing functions will likely have a more significant effect on the drug's renal clearance. Renal clearance represents the sum of glomerular filtration and tubular secretion, adjusted for tubular reabsorption. In renal disease these processes may not decline in parallel due to compensatory hyperfiltration of the remaining intact nephrons. Furthermore, hypoalbuminemia, hyperlipidemia, azotemia, and other secondary effects of renal disease can alter drug protein binding and distribution, in turn influencing the rate and extent of tubular secretion, particularly of highly proteinbound basic drugs [104,105]. Because of the complexity of factors influencing drug disposition in renal disease, specific consequences for PN 200-110 were not predictable, despite the known effects of impaired kidney function on other dihydropyridines [101,102]. However, given the overriding effect of reduced glomerular filtration rate (GFR) on drug disposition, the

following study determined the effect on PN 200-110 pharmacokinetics using GFR as the principal measure of renal dysfunction.

In a single-period parallel group design 23 subjects, stratified by their varying degrees of GFR (creatinine clearance mL/min <10, n = 10; 1130, n = 3; 3180, n = 9; >80, n = 10) each received a single 10 mg capsule of PN 200-110. Serial blood samples were obtained 048 h postdose. During this evaluation period no hemodialysis was performed [106]. To ensure safety and reduce confounding variables, renally impaired subjects with acute or clinically unstable renal disease, accelerated or secondary hypertension other than renal hypertension, and congestive heart failure were excluded from the study. Antihypertensive therapy other than dihydropyridines was continued as required, precluding an assessment of pharmacodynamic effects in conjunction with the pharmacokinetic effects of renal impairment. Bioavailability comparisons were based on the various derived parameters as described in the previous pharmacokinetic studies in this chapter. The mean plasma level data for the 23 subjects are shown in Figure 12. There were no statistically significant differences in the mean bioavailability or pharmacokinetic parameters between subjects with normal renal function (mean GFR 114 ml/min, range 81141) and patients with mildly

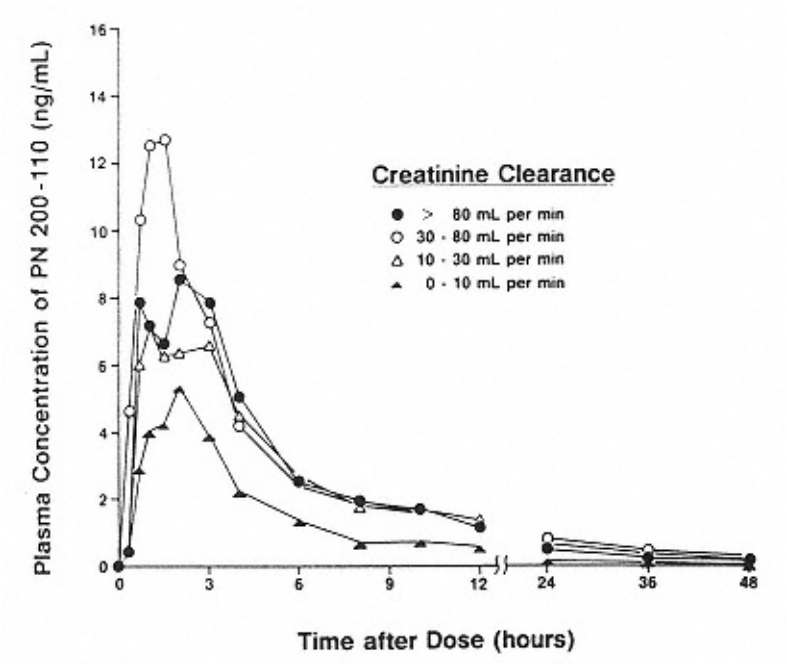

Figure 12
Mean plasma levels of PN 200-110 in subjects grouped by varying renal function.

(mean GFR 50 ml/min, range 3773) or moderately (mean GFR 20 ml/min, range 1126) impaired renal function. The trend toward higher PN 200-110 levels in the mildly impaired subjects may be a result of their higher age (mean 37 years, range 2754) than the control subjects (mean 25 years, range 2032). This could not be conclusively established by multifactorial analysis and illustrates the need for carefully matched groups when a parallel study design is used. Severely impaired or anuric patients (GFR 010 mg/ml) showed a statistically significant reduction (~50% of normals) in AUC and C_{max}.

From this study it would appear that severely renally impaired or anuric patients may require somewhat higher dosages of PN 200-110 to achieve drug levels comparable to those seen in subjects with more normal renal function. Patients with moderate or mild renal impairment should not require specific dosing recommendations. Since hemodialysis may remove circulating drug or metabolites, its use was contraindicated during the evaluation periods of the renal insufficiency study with PN 200-110. It was determined later in a separate study that PN 200-110, in contrast to some other calcium channel blockers, is not removed by hemodialysis and that supplemental doses after hemodialysis are not necessary [106]. Particularly for new drugs with a potential for abuse or those with a small threshold between therapeutic and toxic concentrations, the effectiveness of hemodialysis should be evaluated in a separate pharmacokinetic study.

3. Effect of Hepatic Impairment on PN 200-110 Pharmacokinetics

For drugs like PN 200-110 that are subject to significant metabolism and/or elimination by the liver, the effect of hepatic dysfunction on pharmacokinetics and bioavailability may be substantial and of possible clinical significance. Because hepatic dysfunction can have a parenchymal, vascular, or hepatobiliary basis and the underlying causes and pathogenic mechanisms often are obscure, its total influence on drug disposition is unpredictable. Thus, pathological processes in the parenchyma can lead to chronic decrease in the metabolic function of hepatocytes. The disordered liver architecture seen in cirrhosis can cause increased resistance in the hepatic vascular bed, portal venous hypertension, and portal-systemic venous shunting, with an attendant reduction in the first-pass metabolism of highly extracted drugs. Biliary involvement may affect the drug's and drug metabolites' conjugation and excretory processes [107,108]. This suggests the need for a study of these effects at an appropriate time during the NCE's development, but also points to the importance of careful study design and thorough evaluation of the patients involved. Unlike renal function assessment based on dynamic or indirect determination of GFR by inulin or creatinine clearance, no single conventional biochemical marker or dynamic test provides comprehensive information on the severity of liver dysfunction [109]. However, as seen in the following study with PN 200-110, when used in combination such

tests can type and quantitate liver dysfunction to such an extent as to provide some predictive value for the disposition of drugs in hepatic impairment [110].

Fifteen subjects (10 M, 5 F), mean age 46 years (range 2064) with biopsyconfirmed chronic active hepatitis (n = 7) or cirrhosis (n = 8), and characterized by conventional and dynamic liver function tests, received a 10 min intravenous infusion of carbon 13 (stable isotope labeled) PN 200-110. At the end of the infusion nonlabeled PN 200-110, 5 mg orally as 2 × 2.5 mg tablets, was administered. A control group of normal healthy male subjects, mean age 34 years (range 2050) received the identical treatment. Blood samples for plasma PN 200-110 determinations by GC/MS [56] were obtained prior to and after the end of the infusion up to 28 h. The GC/MS method allowed for the simultaneous determination of standard nonlabeled and stable-isotope-enriched PN 200-110. The derived parameters AUC, C_{max}, volume of distribution, clearance, and the absolute bioavailability calculated from the dose corrected AUC ratios for orally and intravenously administered PN 200-110 were compared between the normal and liver-impaired subjects and also were correlated with the various liver function measures. These included the dynamic function tests of galactose elimination capacity, aminopyrine breath test, and indocyanine green clearance and the indirect serum markers of liver enzymes, creatinine, albumin, and bile acids.

The absolute bioavailability of PN 200-110 was significantly increased from 16% in control subjects and 17% in patients with noncirrhotic chronic liver disease to 37% in cirrhotic patients. This was parallelled by a decline in the systemic clearance from 0.91.14 L/min in control and noncirrhotic patients to 0.6 L/min in the patients with cirrhosis. The plasma clearance of PN 200-110 after i.v. and oral administration was positively and significantly correlated with the disappearance rates of indocyanine green, a measure of hepatic perfusion, and aminopyrine, a measure of hepatic microsomal function. There was no convincing correlation of PN 200-110 clearance with galactose elimination, a measure of functional liver cell mass, with serum bile acid concentrations, a measure of portosystemic shunting, or with the other serum markers.

This study showed that hepatic perfusion and metabolizing capacity influenced the disposition of PN 200-110, which should be expected for this drug based on its first-pass metabolism and structural similarity to other dihydropyridines [111]. The study results corroborated the general conclusions from other studies that dihydropyridines should be used cautiously in patients with cirrhosis. However, as was also the case for the renal insufficiency study with PN 200-110 (see Section V.C.2), specific individual recommendations for dosage adjustments in the impaired population are not readily predictable from such a study. The control group of eight subjects in the liver

insufficiency study showed mean AUC, adjusted to a 10 mg dose of 31 ± 4.4 ng·h/mL, which was well representative of the AUCs from 57 other normal male volunteers (see Fig. 11). The mean oral AUCs adjusted to 10 mg dose for the cirrhotic patients were 103

ng·h/mL (range 22180, with 5 of 8 patients within AUC range 2294, 3 of 8 patients within AUC range 147180) attesting not only to higher but also to extremely variable bioavailability in patients with cirrhosis compared to normals. Thus, the majority of cirrhotic patients showed AUCs comparable to those observed in normal elderly subjects. The three patients with extreme results did not differ consistently from the other cirrhotic patients in terms of their demographics or standard or functional test data.

Practical dosing recommendations consistent with the variable results of this liver insufficiency study would be to expose cirrhotic patients requiring antihypertensive medication to an initial regimen of 2.5 mg/day PN 200-110 with careful monitoring of appropriate safety and efficacy variables, and subsequent dosage escalations to 5 mg/day or higher as required for efficacy upon assurance of the safety of the previous dosing schedule.

The intravenous data generated in normal volunteers in this study allowed for calculation of plasma clearance, steady-state volume of distribution, and, in conjunction with the oral data, the fraction of orally bioavailable drug. Absorption per se, that is, the fraction of the administered oral dose crossing the gastrointestinal barrier into the systemic circulation either as unchanged drug or metabolites, is best determined from an ADME study using radiolabeled drug (see Section III.C.2). In the absence of intravenous data, noncompartmental determinations of these parameters can be obtained from the oral data, employing areas and area moments for the calculation of Vd_{ss} [112,113], whereas the fraction of the orally administered drug reaching the systemic circulation intact can be derived from hepatic blood flow rate considerations [114,115]. The mean fraction PN 200-110 bioavailable calculated from the oral AUC/i.v. AUC ratios of the 8 control subjects in the hepatic insufficiency study was 17 ± 10% [110]. This compares well with mean values of 18% calculated from the single-dose ADME study (see Section III.C.2) and the 19 ± 7% calculated using the above summarized procedures for the oral data from 57 normal subjects pooled from several bioavailability studies (see Fig. 11).

4. Effect of Concomitant Medication on PN 200-110 Pharmacokinetics

It is very common for patients to receive two or more drugs concomitantly. There is also the possibility that some of the drug combinations can interact adversely. From a pharmacokinetic standpoint, these interactions can involve an alteration in the absorption, distribution, metabolism, or excretion of one or both compounds. Since it is not possible to study all potential drug interactions for a new drug under development, judicious choices must be made about which particular combinations will be evaluated. Selection is normally limited to drugs likely to be prescribed simultaneously by clinicians or relevant over-the-counter medications. Thus for PN 200-110, its potential pharmacokinetic

interaction with hydrochlorothiazide was investigated [116].

Using a 3 × 3 replicated Latin square design, 21 volunteers were administered 10 mg capsules of PN 200-110 or 50 mg hydrochlorothiazide (HCTZ) tablets, either individually or concomitantly. Each administration was separated by a 510 day washout period. Serial blood samples were obtained between 0 and 30 h following each dosing period and plasma concentrations of PN 200-110 and HCTZ were determined. The mean data for the 21 subjects are shown in Figure 13. The bioavailability parameters evaluated for this study were the same as those discussed previously for other pharmacokinetic studies. For PN 200-110 there were no statistically significant differences in the bioavailability parameters on concomitant administration of HCTZ compared to PN 200-110 alone. Differences in AUC and C^{max} were <10%. Similar results were observed with HCTZ, for which differences for AUC and C^{max} were <5%. Statistical power was adequate to detect 20% or greater differences in these parameters for either drug. These results demonstrate that concomitant administration of PN 200-110 and HCTZ does not result in altered pharmacokinetics of either agent.

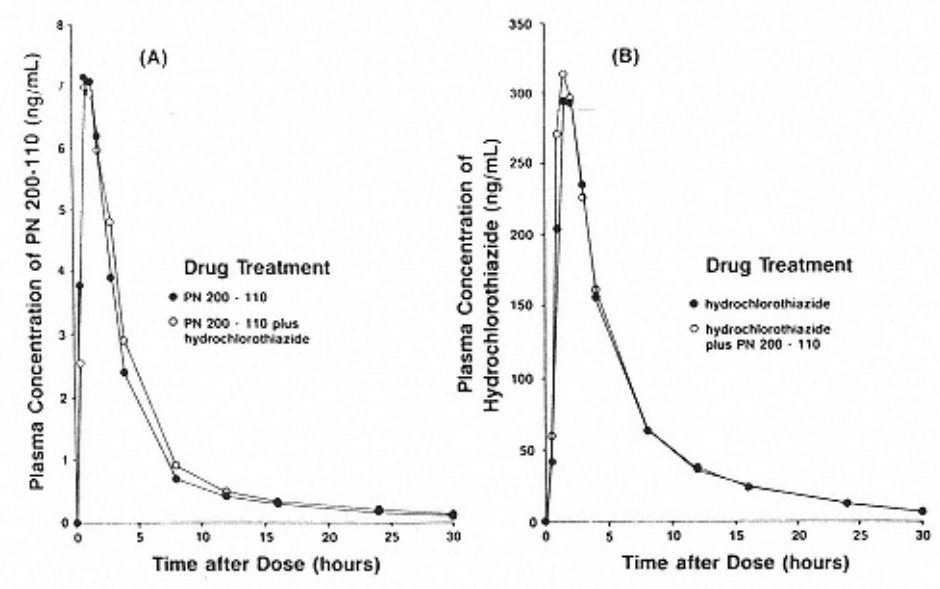

Figure 13

(A) Mean plasma levels of PN 200-110 following administration of 10 mg PN 200-110 or 10 mg PN 200-110 plus 50 mg hydrochlorothiazide. (B) Mean plasma levels of hydrochlorothiazide following administration of 50 mg hydrochlorothiazide or 50 mg hydrochlorothiazide plus 10 mg PN 200-110.

Because pharmacokinetic drug-drug interactions often are caused by specific inductive or inhibitory effects of the one drug on the enzyme systems responsible for the biotransformation of the other drug, in vitro screening for interferences in the cytochrome P450 or other metabolizing systems provides a useful assessment of potential interactions. Knowledge of the enzyme systems involved in drug metabolism can be obtained from studies with cell cultures, tissue slices, or animal models. These systems can then be used to elucidate the mechanism of previously observed drug-drug interactions or detect potentially interfering agents [117].

5. Effect of Food Intake on PN 200-110 and OG 37325 Pharmacokinetics

It has been well-established that meals, especially those with a high fat content, can have a profound effect on the rate and extent of absorption of drugs [118]. Food interaction studies have become an integral part of most NDA submissions. This type of study was of particular importance for PN 200-110, since nifedipine, a drug with a similar structure (dihydropyridine) and pharmacological class (antihypertensive), had previously been shown to have a significantly delayed and lower C_{max} when administered with food compared to the fasting state [119,120].

In a two-period crossover design a panel of 16 normal volunteers received single oral doses of PN 200-110 capsules on a fasted stomach or with a highfat breakfast [120]. Prior to each dosing day, subjects received an evening meal, then fasted for at least 12 h. Water was available ad libitum. At approximately 8 a.m. the following morning one group of 8 subjects received a single 10 mg PN 200-110 capsule with 2 oz water on a fasted stomach. The standard highfat breakfast was administered 2 h postdose. The other group of 8 subjects ingested their breakfast and took a single 10 mg PN 200-110 capsule approximately 10 min after completing the meal, at about 8 a.m. A light midday snack was served at about 1 p.m. and the evening meal at about 6 p.m. All subjects received the same meals. The two study periods were separated by a 7 day washout period, with the treatments reversed for the two groups in study period 2. Serial blood samples were obtained 015 h postdose. Bioavailability parameters were obtained as previously described for other studies in this chapter. Following administration of PN 200-110 on a fasted stomach, the drug was rapidly absorbed, with peak plasma levels achieved after 1.4 h. Administration of the drug with food resulted in a statistically significant increase in the lag time to absorption by about 50 min and consequently a delay in the time to peak of about 50 min. Peak concentrations (C_{max}) and the extent of bioavailability, as reflected by the AUC, were unchanged, as shown in Figure 14. The small secondary peak in the mean plasma level curve following administration of drug with food is an artifact from averaging the individual data, in which secondary peaks were not discernible and

pointing to the absence of enterohe-

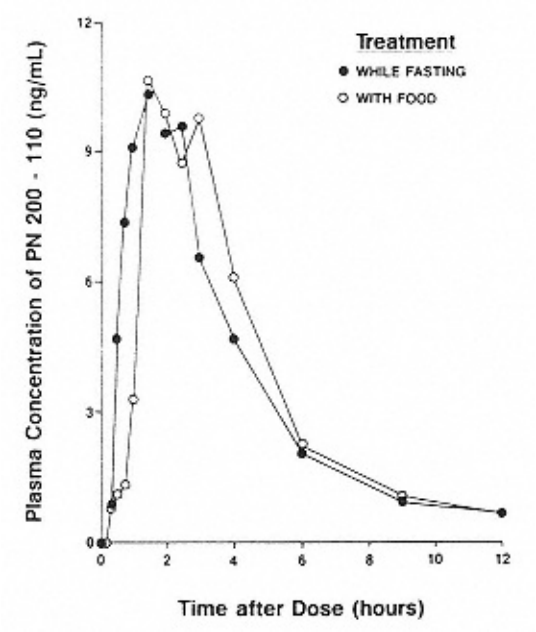

Figure 14
Mean plasma levels of PN 200-110 following administration of the drug with food or in the fasting state.

patic recycling or other processes associated with such peaks. Thus, the administration of PN 200-110 with food has no appreciable effect on bioavailability. This means that dosing restrictions with respect to food intake are unnecessary for this drug.

Drug absorption is a complex process governed by the interaction of the drug's physicochemical and pharmaceutical profile with such physiological variables as gastrointestinal pH, water flux, hepatic and gut wall drug-metabolizing enzymes, secretion of bile, gastrointestinal motility, splanchnic blood flow, and microbial activity in the intestine [118,121,122]. In the presence of food these interactions are variedly perturbed, depending upon the size and composition of the meal and its time of ingestion in relation to drug intake. Changes in gastric residence time, which depend mainly on food composition, particularly fat content, frequently affect the rate but not necessarily the extent of drug absorption, as was exemplified by the PN 200-110 capsule, which showed delayed time to peak concentrations but no appreciable change in the AUC. Assessing potential food effects with the high-fat FDA-type breakfast [120] in comparison to the

fasted state (of >10 h) explores the probable extreme range of the potential food interaction in a simple study design. Although it is unlikely that breakfast administered 2 h after drug ingestion, as was done for the PN 200-110 food interaction study, would interfere in the drug absorption process, it has been recommended that the fasting period be extended to 4 h postdose for drugs with erratic absorption or sustained-release drug products [123]. For those drugs for which the effect of food on absorption could be problematic, a study design should be considered that examines bioavailability when the drug is administered at fixed time points before, during, and after the meal. Knowledge of the effect of variable meal size or composition on drug bioavailability may likewise be desirable for drugs sensitive to food effects. This was investigated in some detail with a solution and an experimental soft gelatin capsule of OG 37325. Prior experience with the structurally similar drug, cyclosporin A (see Fig. 1), had shown variable effects of food, resulting in either increased [124] or decreased bioavailability [125], prompting the need for a more detailed exploration of the food effect on both formulations of OG 37325.

Two groups of normal healthy male volunteers, comparable with respect to age, height, weight, and race, received single oral 225 mg doses of OG 37325 as 2.25 ml of the solution or 3 × 75 mg soft gelatin capsules with meals of differing composition or on a fasted stomach. Each group received in random order the following five treatments over five periods of this 5 × 5 crossover study:

Group 1 (n = 15 Subjects)	*Group 2* (n = 15 Subjects)
Capsules, fasted	Solution, fasted
Capsules, low-fat (6 g) breakfast	Solution, low-fat (6) breakfast
Capsules, moderate-fat (30 g) breakfast	Solution, moderate-fat (30 g) breakfast
Capsules, high-fat (80 g) breakfast	Solution, high-fat (80 g) breakfast
Solution, moderate-fat (30 g) breakfast	Capsules, moderate-fat (30 g) breakfast

The linkage between the two groups, representing the two formulations given with a meal of moderate fat content, was chosen as most representative of the expected dosing conditions, with the drug given with breakfast and evening meals. Blood concentrations of OG 37325 were determined to 48 h postdose by HPLC. Bioavailability comparisons were based on AUC, C_{max}, and t_{max} of the blood concentration vs. time curves. The solution administered with a moderate-fat breakfast was taken as the standard for the comparison; the other treatments showed the following relative bioavailability:

Parameter Comparisons for Group 1

	Mean Parameter Ratio		
	AUC	Cmax	tmax
Capsules, fasted	0.46	0.62	1.6
Capsules, low-fat	0.68	0.83	1.5
Capsules, moderate-fat	1.02	1.07	1.7
Capsules, high-fat	0.93	0.95	1.4
Solution, moderate-fat[a]	1	1	1

Parameter Comparisons for Group 2

	Mean Parameter Ratio		
	AUC	Cmax	tmax
Solution, fasted	0.86	1.04	0.9
Solution, low-fat	0.99	1.06	0.9
Solution, moderate-fat[a]	1	1	1
Solution, high-fat	0.92	0.82	1.1
Capsules, moderate-fat	0.87	0.91	1.5

[a]Used as standard

There was no appreciable food effect with the OG 37325 solution. In contrast, there was a pronounced loss of bioavailability with the capsule taken on a fasted stomach. However, with increasing fat consumption bioavailability of the capsule was restored, so that taking the capsule with a meal of moderate fat content produced nearly identical AUC and C_{max}, although delayed t_{max}, as the solution. Thus, dosing guidelines regarding administration of the capsule with food would be desirable to ensure adequate bioavailability.

D. Population Pharmacokinetic Screen with PN 200-110

In all the previously discussed studies of PN 200-110, average pharmacokinetic parameters were obtained from small, relatively homogeneous groups of volunteers or patients. Collectively these studies define the general pharmacokinetic profile for PN 200-110. The Biopharmaceutics Chapter of an NDA (Chapter VI, see Table 1) presents a summary of the individual pharmacokinetic parameters from all such studies, grouped by population, formulations administered, or by other study variables. In this way population means and other descriptive statistics can be determined for relevant groupings across studies, which can include several hundred volunteers or patients. A major, clinically relevant purpose in determining this profile is to develop dosing guidelines for specific populations who may be at particular risk when taking the drug. The disadvantage of this approach is that the overall pharmacokinetic profile elaborated from specific studies is limited to the various patient characteristics and underlying pathology, which were a priori assumed to be relevant for all future patients taking the drug. In an alternative approach, that of the prospective pharmacokinetic screen, the population, rather than the individual, serves as the unit of pharmacokinetic analysis [2227,126]. By evaluating PN 200-110 levels in a large number of patients a more representative sample of the target population was accessible, even though far fewer data points per patient are normally available in such population pharmacokinetic studies than

in the pharmacokinetic studies discussed previously. In the course of seven clinical phase III studies

assessing the safety and efficacy of orally administered (520 mg/day) PN 200-110, plasma samples were obtained during routine clinic visits from hypertensive patients taking the medication for blood pressure control. Sampling was performed at any convenient time point after the morning dose but before the second dose of the twice daily regimen of the drug in capsule formulation. No restrictions with respect to sampling frequency, number or type of patients sampled, concomitant medications, or other variables were made. Of the nearly 1500 plasma samples collected in the course of the study, 735 samples from 263 patients who had complied with their assigned dosing regimen were available for evaluation (samples from suspected noncompliers were also evaluated for metabolite concentrations; all subjects showing no measurable parent drug and metabolite concentrations and those on placebo control were excluded). In comparison, the phase II population pharmacokinetic study with ZK 112119 yielded about 30% of these patient and sample numbers (see Section IV.B). Figure 15 depicts a plot of the dose-normalized PN 200-110 concentrations in these 735 specimens as a function of time after the morning dose.

Several computational techniques have been advocated for the evaluation of population pharmacokinetic data [2224] and are also discussed in Section IV.B. Since the major focus of the population screen for PN 200-110 levels was to identify subgroups of patients differing from the majority and to identify those factors possibly contributing to the differences in PN 200-110 levels, the approach taken was to group individual data into the classifications low, middle, and high plasma levels using cluster analysis [87], analogous to the quartile approach used for the ZK 112119 population kinetic evaluation. Figure 15 depicts these data groups. The groups were compared with respect to demographic variables, clinical laboratory data, and frequency of adverse reactions encountered in the course of the study. Of the demographic data, only age showed significant differences among groups: a higher age being associated with higher PN 200-110 levels, a finding corroborated in the separately performed labeling study discussed previously (see Section V.C.1). Mean clinical laboratory parameters showed no statistically significant differences between groups. There were no differences in the frequency of adverse reactions between groups.

As discussed for the phase II population kinetics screen with ZK 112119, the collection of large numbers of blood specimens in the course of clinical trials, particularly under conditions that ensure the integrity of drug analyte, presents great logistical difficulties. The intent of a population pharmacokinetic study is to search for the unexpected, unusually high or low drug level achieved with a given dose. The presumably increased likelihood of detecting unusual responders in the larger data base provided by a phase III over a phase II population screen must be weighed against the possible deterioration in

quality of the larger data base due to logistical problems. Similar considerations apply when judging the value of a population pharmacokinetic screen, whether phase

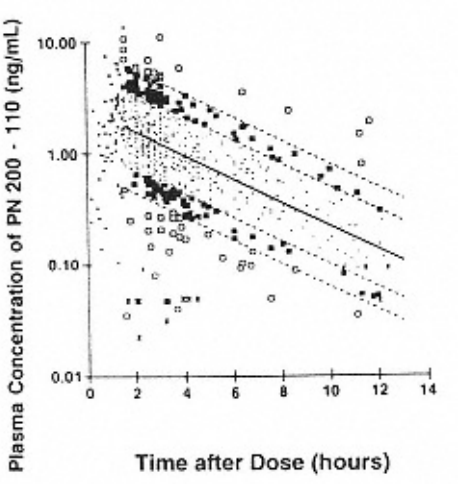

Figure 15
Individual (dosage normalized) PN 200-110 levels in 263 hypertensive patients evaluated in a population pharmacokinetic screen: values for upper and lower 10th percentile (□), 10 15th percentile (▪), and mid-70th percentile (•) of values in the time interval 1.512.5 h postdose; values in the absorption phase (01.5 hr) (○) and values below the limit of quantification of the analytical method (x) were excluded from subsequent analysis. The solid line represents linear regression of data points 1.512.5 h postdose.

II or phase III, against separately conducted labeling studies in volunteer or patient groups. Furthermore, the value of a population pharmacokinetic study over separate labeling studies for a given drug will have been demonstrated only if the population screen was successful, in that the study was able to characterize by convincing statistical criteria such subpopulations of patients showing excessive or subtherapeutic drug levels who were not readily identifiable in the standard labeling studies described above. Thus it is unclear if the evaluation of 735 plasma samples from 263 patients in the phase III PN 200-110 population pharmacokinetic study was sufficient to identify all groups potentially at risk. Argument for or against either approach, that of specific studies vs. a population pharmacokinetic screen, should therefore be reconciled towards designing a drug development program that combines both the labeling studies and the population pharmacokinetic program as representing the most effective approach to define specific subpopulations at risk. The timing for conducting the population screen, during clinical phase II or phase III, depends on the specific requirements of the drug under development and may be determined in part by the total number of patients available for screening in phase II [127].

E. Concentration-Controlled Clinical Trial with Cyclosporin A

The rationale, concept, and regulatory viewpoint regarding RCCT have been presented in several publications [82,84,128]. In a standard, dose-controlled clinical trial, comparisons between treatment groups can be complicated by pharmacokinetic variability, necessitating larger panel sizes to discern treatment differences and possibly precluding the thorough and safe exploration of the higher end of the therapeutic dosage range. In the RCCT, patients are assigned to predetermined levels of average drug concentration and subsequently maintained within their target concentration range. This should minimize pharmacokinetic variability within and between the treatment groups, leading in turn to decreased variability in the clinical response with attendant advantages of panel size efficiency and safety. The underlying assumption is that drug plasma concentrations are more closely related to drug response than drug dosage. Using the pharmacodynamic model for theophylline, it has been determined that improvements in the panel size efficiency of the RCCT are more sensitive to decreases in pharmacokinetic than pharmacodynamic variability [82]. These improvements can be realized in RCCT approaches involving parallel groups with or without placebo control in a fixed or escalating concentration design [84].

The attractive theoretical concept of the RCCT must be weighed against its practical difficulties, such as establishing a clinically acceptable frequency of blood draws consistent with study objectives; appropriate sample handling protocols for the clinic and bioanalytical site to ensure analyte integrity; availability of a validated bioanalytical method providing adequate turnaround of data; mechanisms to ensure continued blinding of the trials; competency to exploit Bayesian adaptive dosing schemes based on the drug concentration data so as to minimize the need for blood sampling. These practical considerations are reminiscent of those guiding the conduct of the population pharmacokinetic trials discussed in Sections IV.B and V.D, although the prospective nature of the RCCT imparts greater urgency and a higher investment of supporting resources for generating the drug concentration data.

The drug cyclosporin A (cyclosporine, Sandimmune; see Fig. 1) is an immunosuppressant widely used to improve graft acceptance in patients undergoing organ transplantation [31,129]. It is one of approximately 20 drugs that have a defined therapeutic range and for which routine drug concentration monitoring is widely used [130]. Dosage optimization based on whole blood cyclosporin concentrations at trough by HPLC or specific immunoassay attempts to maintain patients within the range that prevents transplant rejection due to subtherapeutic concentrations and protects from nephrotoxicity or other adverse events resulting from excessive drug concentrations [129,131]. This is

complicated by the considerable pharmacokinetic variability already evident after administration of sin-

gle oral doses to normal subjects [124,132] and very pronounced in patients at or achieving the steady state [125]. In addition to its use in patients undergoing organ transplantation, cyclosporin A has shown clinical benefit in diseases of probable autoimmune cause including psoriasis and rheumatoid arthritis (RA) [133,134]. For these autoimmune indications the upper therapeutic range is set by dosage or concentration-limited adverse events, with nephrotoxicity being of particular concern for patients with RA. The lower therapeutic range, not being limited by considerations of organ rejection, is less critical than for the transplantation indication. Nevertheless, choosing a clinically relevant individual starting dosage and an appropriate rate and degree of dosage escalation of cyclosporin A in patients with RA presents the challenge of weighing an aggressive regimen with immediate clinical benefit but potential for nephrotoxicity [133] against an approach of low starting dosage and conservative dosage escalations with its potential for treatment failure or premature discontinuation of medication [134]. Therefore, an RCCT approach was selected to explore the duration of drug exposure at a specific concentration and the escalation schedule that would provide optimal clinical benefit for patients with RA. Male and female patients who had failed to respond to at least one established second-line therapeutic agent for RA were enrolled in this double-blind, placebo-controlled study. Patients were assigned to an initial dosage group: 0, 1.5, 2.5, or 4.5 mg/kg/day (given in divided doses every 12 h). These dosages were chosen based on previous experience as providing the greatest likelihood for patients to achieve the desired target concentrations. Within the subsequent 2 weeks patients' dosages were titrated to achieve target (allowable range) cyclosporin A concentrations in blood of 0, 50 (4060), 100 (80120) and 175 (140220) ng/ml, respectively. An unblinded investigator instructed the blinded investigator as to appropriate dosage adjustments, including random adjustments of comparable magnitude for the patients receiving placebo. During study weeks 28, downward titrations were allowed for safety reasons; study weeks 814 allowed both downward and upward titration, the latter to optimize efficacy. An open-label extension of the study was provided for patients to continue beyond study week 14. Efficacy was assessed based on standard objective and subjective criteria, including patient and examiner global assessment, changes in joint size and joint involvement, and other such measures of RA status. Safety was assessed from medical examination, vital signs, and clinical laboratory data. A total of 191 patients were entered into the study, similarly balanced across the four concentration groups, with 140 completing the 14 week treatment. Distribution of the dropouts across the 0, 1.5, 2.5, and 4 mg/kg groups was 16, 6, 3, and 1 patients, respectively, for lack of efficacy, and 0, 1, 6, and 6, respectively, for adverse reactions, with the remaining 12 patients discontinuing for various reasons not related to study medication. This indicates a rough positive relationship of adverse reactions and efficacy with increased dosage or concentra-

tion. However, the relationship of dosage or drug concentration with various efficacy or safety measures was not significant for either the intent to treat analysis group, representing the 191 patients who entered the study and provided efficacy follow-up data, or the valid completers group, representing the 140 patients who stayed on study medication for at least 12 of the 14 study weeks. Despite careful attention to logistical details and specific dosing instructions to the clinical investigators for achieving the desired drug concentrations, only 21 of the 140 valid completers remained consistently within their assigned target ranges, precluding a statistical analysis. Retrospective grouping of the completers into three target concentration ranges of 50 (3570), 100 (71130), and 175 (>130) ng/ml was possible for 68 of 140 (49%) of patients. The lowest concentration group showed no difference from placebo in safety or efficacy parameters. Both the medium and high concentration group showed significant clinical improvement compared to placebo at the majority of the post week 4 evaluations although, unexpectedly, the greatest improvement was consistently associated with the medium, and not the high, concentration group. Retrospective analysis of the completers' data as two drug concentration groups of <120 and >120 ng/ml also revealed efficacy for both groups compared to placebo but again without indication of ranking with respect to concentration. Excursions in serum creatinine >30% from baseline values, an indication of cyclosporin A nephrotoxicity, were most prevalent in the highest drug concentration group. However, the correlation of individual drug concentrations with creatinine changes was statistically no stronger than the correlation of drug dosage with creatinine. There was no significant association of blood pressure elevations or other drug-associated side effects with drug concentration range or drug dosage.

This study revealed no outstanding advantage of concentration control for the conduct of a clinical evaluation of cyclosporin A in patients with rheumatoid arthritis. The study showed that the logistical problems associated with performing an RCCT, of sample collection, drug concentration analysis, and use of the data while maintaining the study blind, can be successfully addressed, albeit with a considerable investment of resources. However, since the potential advantages for sample size efficiency and statistically better defined results were not realized in this study, its lesson is that the cost vs. expected benefit for pursuing the RCCT approach must be carefully weighed, on a case-by-case basis, for every new drug in development. For the use of cyclosporin A in RA, the study revealed that between a starting dosage of 1.5 mg/kg/day and maximum dosages not normally to exceed 45 mg/kg, clinical benefit in the absence of unmanageable side effects can be achieved,with the expectation of slow and gradual course in clinical improvement that should not be accelerated with early aggressive dosage escalations or be misinterpreted as treatment failure.

VI. Post-NDA Studies

The studies presented as examples in this chapter represent a basic pharmacokinetics package that should meet present FDA requirements for an NDA submission. However, it is often necessary to perform additional pharmacokinetic studies, either during the clinical development process (phases I through III) or after the drug has been approved for marketing. The postapproval stage is frequently referred to as phase IV. Typical trials in phases I-IV additional to those discussed above may include the evaluation of alternative routes of administration, of improvements in the dosage form described in the original NDA, or of alternative dosage forms that modify the release of the drug compared to the conventional oral form. The evaluation of new indications for drug use can also necessitate further special pharmacokinetic (labeling) studies. As previously discussed, an amendment to the original IND or a new IND submission may be required to conduct such pharmacokinetic studies, and upon their completion either a new NDA or an NDA amendment may have to be filed. The scope of the pharmacokinetic studies will depend upon the changes sought from the original NDA. Thus, for example, regulatory approval of a modified-release version of an approved oral form requires a detailed pharmacokinetic evaluation after single oral doses and at steady state in comparison to the approved form, as well as an assessment of the effect of environmental factors, such as food intake, on the performance of the modified-release form. It is unlikely in today's regulatory climate that approval can be obtained solely on the basis of such pharmacokinetic studies, and thus additional clinical studies to evaluate safety and efficacy may be prudent. In contrast, for a new formulation of an approved drug that makes no claims of a controlled or modified release, or any other claims not contained in the original NDA, a single-dose bioavailability study should suffice for regulatory approval. In a typical study the experimental form is compared to the marketed form in fasted normal volunteers using the standard two-way crossover design discussed in Section III.C.3. The previously discussed criteria for bioequivalence and statistical acceptability apply. The data for this study and appropriate revisions to the original drug labeling can be submitted in the form of an abbreviated NDA (ANDA) or a supplement to the original NDA.

VII. Conclusions

From the discussions in this chapter it is apparent that pharmacokinetic studies play a vital role in the clinical drug development process. Although the suggested package of studies outlined in Table 2 should be adequate for a standard biopharmaceutics submission based on present federal regulations and guidelines, there is no guaranteed standard approach that can be utilized for all

NDA submissions. All drug candidates are different and therefore pharmacokinetics must be used as a flexible tool that can aid the clinical researcher in dealing with the wide range of questions and uncertainties encountered during the NDA process. Approached in this way, pharmacokinetic studies can substantially decrease the risk to subjects (blood level monitoring), isolate subpopulations that should be dosed differently, aid in the design and evaluation of clinical trials, and contribute to the development of more effective dosage forms. Thus, if correctly applied, pharmacokinetics can play an important role in the development of safer and more effective drug therapy. Essential elements for judicious application are carefully planned pharmacokinetic studies. Since these studies are performed throughout the drug development process, which usually takes many years to come to fruition, this poses a major challenge to the individuals responsible for their design, scheduling, and execution. In particular, a changing regulatory climate, as well as continuing scientific progress, render long-term planning difficult. Continued dialogue between workers in the field of drug development and the regulatory agencies, as well as discussions as exemplified in this volume, should assist in facilitating this process.

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References

1. New Drug, Antibiotic, and Biologic Drug Product Regulations; Final Rule, Section 312.3. Definitions and Interpretations. Fed. Reg. 52 (No. 53):87988847, 1987.

2. U.S. Code of Federal Regulations, Title 21, Protection of Human Subjects; Standards for Institutional Review Boards for Clinical Investigations, Parts 56.10156.124 (Docket No. 77N-0350); see also 21 CFR 312.66, Assurance of IRB Review [Docket No. 82N0394]; see also D. H. Cowan, Issues in the review of clinical drug trials by IRB's, in Clinical Drug Trials and Tribulations. Edited by A. E. Cato. Marcel Dekker, New York, 1988.

3. P. F. D'Arcy and D. W. G. Harron (eds). Proceedings of the First International Conference on Harmonization. Queen's University, Belfast, Ireland, 1992.

4. W. J. C. Currie. Drug development and registration in Japan: Threshhold of transition.

J. Clin. Pharmacol. 33:100108, 1993.

5. V. Skeffington. EC legislation for human medicinal products: Guidelines parts 1 and 2. Reg. Affairs J. 4:706709, 727733, 798801, 1993.

6. U.S. Code of Federal Regulations, Title 21, New Drugs for Investigational Use, Part 312.1 (a.2), Special Edition of Federal Register. U.S. Government Printing Office, Washington, D.C., 1981, pp. 7074.

7. D. B. Campbell and R. M. J. Ings. New approaches to the use of pharmacokinetics in toxicology and drug development. Human Toxicol. 7:469479, 1988.

8. U.S. Code of Federal Regulations, Title 21, Bioavailability and bioequivalence requirements, Parts 320.1320.62, Special Edition of Federal Register. U.S. Government Printing Office, Washington, D.C., 1981, pp. 120138.

9. J. M. Jaffe and H. F. Schran. Clinical pharmacokinetics in drug discovery and development. In Pharmacokinetics: Regulatory, Industrial, Academic Perspectives. Edited by P. G. Welling and F. L. S. Tse. Marcel Dekker, New York, 1988.

10. A. Yacobi, V. K. Batra, R. Desjardins, R. Faulkner, G. Nicolau, et al. Implementation of an effective pharmacokinetics research program in industry. In Integration of Pharmacokinetics, Pharmacodynamics, and Toxicokinetics in Rational Drug Development. Edited by A. Yacobi, J. Skelly, V. Shah, and L. Benet. Plenum Press, New York, 1993, pp. 125136.

11. C. C. Peck, W. H. Barr, L. Z. Benet, et al. Conference Report: Opportunities for integration of pharmacokinetics, pharmacodynamics, and toxicokinetics in rational drug development. Pharm. Res. 9:826833, 1992.

12. U.S. Code of Federal Regulations, Title 21, Investigational new drug application, Subpart B, Responsibilities of sponsors, Part 312.50312.59 [Docket No. 82N0394]; also see 21 CFR 10.90 Guideline for the monitoring of clinical investigations, Jan. 1988, [Docket No. 82D-0322], Bioresearch Program Coordinator (HFC-230), Food and Drug Administration, Rockville, MD.

13. U.S. Code of Federal Regulations, Title 21, Good Laboratory Practice Regulations, 58.00; also see J. M. Taylor and W. A. Peterson. In Good Laboratory Practice Regulations. Edited by A. F. Hirsch. Marcel Dekker, New York, 1989.

14. Guideline for the format and content of the summary for new drug and antibiotic applications (Docket No. 85D-0247), Food and Drug Administration, Center for Drug and Biologics, Rockville, MD.

15. Guideline for the format and content of the human pharmacokinetics and

bioavailability section of new drug application (Docket 85D-0275), Food and Drug Administration, Center for Drugs and Biologics, Rockville, MD.

16. S. V. Dighe. Current bioavailability and bioequivalence requirements and regulations. Clin. Res. Pract. Drug Reg. Affairs 2:401421, 1984.

17. J. P. Skelly. Bioavailability and bioequivalence. J. Clin. Pharmacol. 16:539545 1976.

18. B. E. Cabana, Assessment of 75/75 Rule: FDA Viewpoint, J. Pharm. Sci. 72:9899, 1983.

19. J. D. Haynes. Statistical simulation study of new proposed uniformity requirements for bioequivalency studies. J. Pharm. Sci. 70:673675, 1981.

20. B. E. Rodda and R. L. Davis. Determining the probability of an important difference in bioavailability. Clin. Pharmacol. Ther. 28:247252, 1980.

21. M. L. Chen, R. Patnaik, S. V. Dighe, and R. L. Williams. Guidance statistical procedures for bioequivalence studies using a standard two-treatment crossover design. Division of Bioequivalence (HFD-650), Center of Drug Evaluation and Research, FDA, Rockville, MD, 1992.

22. C. B. Sheiner and T. H. Grasela. An introduction to mixed effect modelling: Concepts, definition, and justification. J. Pharmacokinet. Biopharmaceutics. 19:115245, 1991.

23. A. H. Thomson and B. Whiting. Bayesian parameter estimation and population pharmacokinetics. Clin. Pharmacokinet. 22:447467, 1992.

24. W. F. Dodge, R. W. Jelliffe, C. J. Richardson, et. al. Population pharmacokinetic models: Measures of central tendency. Drug Invest. 5:206211, 1993.

25. D. M. Kraus, C. M. Dusik, K. A. Rodvold, et. al. Bayesian forecasting of gentamicin pharmacokinetics in pediatric intensive care unit patients. Pediatr. Infect. Dis. J. 12:713718, 1993.

26. J. Grevel, B. K. Part, and B. D. Kahan. Michaelis-Menten kinetics determine cyclosporine steady-state concentrations: A population analysis in kidney transplant patients. Clin. Pharmacol. Ther. 53:651660, 1993.

27. C. L. Devane, T. H. Grasela, E. J. Antal, and R. L. Miller. Evaluation of population pharmacokinetics in therapeutic trials: IV. Application to postmarketing surveillance. Clin. Pharmacol. Ther. 53:521528, 1993.

28. W. A. Colburn. Controversy V: Phase I, First Time in Man Studies. J. Clin. Pharmacol. 30:210222, 1990.

29. C. E. Handler and E. Sowton. Safety, tolerability and efficacy of PN 200-110, a new calcium antagonist in patients with angina and coronary heart disease. Eur. J. Clin. Pharmacol. 27:415417, 1984.

30. T. J. Petcher and H. P. Weber. Conformations of some rigid neuroleptic drugs. J. Chem. Soc. Perkin II:1415, 1976.

31. A. von Wartburg and R. Traber. Chemistry of the natural cyclosporin metabolites. Prog. Allergy 38:2845, 1986.

32. C. Vibelli and G. Rudelli. Ketotifen in bronchial asthma: Results of a multicenter study. Med. Torac. 5:105119, 1983.

33. D. N. Stephens, H. H. Schneider, W. Kehr, et al. Abecarnil, a metabolically stable anxioselective b-carboline acting at benzodiazepine receptors. J. Pharmacol. Exp. Ther.

253:334343, 1990.

34. N. H. G. Holford and L. B. Sheiner. Understanding the dose-effect relationship: Clinical application of pharmacokinetic-pharmacodynamic models. Clin. Pharmacokinet. 6:429453, 1981.

35. V. F. Smolen. Bioavailability and pharmacokinetic analysis of drug responding systems. Annu. Rev. Pharmacol. Toxicol. 18:495522, 1978.

36. G. Levy. Variability in animal and human pharmacodynamic studies. In Variability in Drug Therapy: Description, Estimation, and Control. Edited by M. Rowland. Raven Press, New York, 1985, pp. 125138.

37. M. Montagne. Issues in the design and conduct of clinical trials. Clin. Res. Pract. Drug. Reg. Aff. 3:2344, 1985.

38. S. Bolton. Pharmaceutical Statistics, Practical and Clinical Applications. Marcel Dekker, New York, 1990.

39. Statistical Methodology in the Pharmaceutical Sciences. Edited by D. A. Berry. Marcel Dekker, New York, 1990.

40. E. L. Posvar, A. J. Sedman. New drugs: The first time in man. J. Clin. Pharmacol. 29:961966, 1989.

41. R. B. Merkatz, R. Temple, S. Sobel, K. Feiden, D. Kessler, et al. Women in clinical trials of new drugs. N. Engl. J. Med. 329:292296, 1993.

42. P. L. Bonate. Gender-related differences in xenobiotic metabolism. J. Clin. Pharmacol. 31:684690, 1991.

43. H. T. Karnes, G. Shiu, and V. P. Shaw. Validation of bioanalytical methods. Pharm. Res. 8:421426, 1991.

44. V. P. Shah, K. K. Midha, S. Dighe, I. McGilveray, J. P. Skelly, et al. Conference report; analytical methods validation: Bioavailability, bioequivalence and pharmacokinetic studies. Pharm. Res. 9:588592, 1992.

45. H. T. Karnes and C. March. Precision, accuracy, and data acceptance criteria in biopharmaceutical analysis. Pharm. Res. 10:14201426, 1992.

46. W. J. Dixon and N. Fotheringham. Application of staircase bioassays in pharmaceutical research. Drug Inf. J. 27:741751, 1993.

47. M. Depré, C. Meeter, A. V. Heken, et al. Pharmacodynamics and tolerability of L-654, 066, a steroid 5a-reductase inhibitor, in Men. Clin. Pharmacol. Ther. 52:409412, 1992.

48. J. R. Nedelman, E. Gibiansky, F. L. S. Tse, and C. Babiuk. Assessing drug exposure in rodent toxicity studies without satellite animals. J. Pharmacokinet. Biopharmaceutics. 21:323334, 1993.

49. J. M. Collins, C. K. Grieshaber, and B. A. Chaber. Pharmacologically guided phase I clinical trials based upon preclinical drug development. J. Natl. Cancer Inst. 82:13211326, 1990.

50. P. J. McNamara. Interspecies scaling in pharmacokinetics. In Pharmaceutical Bioequivalence. Edited by P. G. Welling, F. L. S. Tse, and S. V. Dighe. Marcel Dekker, New York, 1991.

51. B. E. Cabana and J. F. Douglas. Bioavailability-pharmacokinetic guidelines for IND development. Paper presented at the 15th Annual International Pharmacy Conference, Austin, Texas, February 2327, 1976.

52. J. R. Jeffery. Cyclosporine analogues. Clin. Biochem. 24:1521, 1991.

53. R. B. Nussenblatt, M. D. deSmet, B. Rubin, et al. A masked, randomized, dose response study between cyclosporine A and G in the treatment of sight-threatening uveitis of noninfectious origin. Am. J. Ophthamol. 115:583591, 1993.

54. A. Leichtman, M. Adams, W. Bennett, et al. OG 37325, a cyclosporine analogue, is safe and effective for the prophylaxis of rejection in human renal transplantation. Presented at the 6th European Congress for Organ Transplantation, Oct. 1993.

55. F. L. S. Tse and J. M. Jaffe. Pharmacokinetics of PN 200-110 (isradipine), a new calcium antagonist, after oral administration in man. Eur. J. Clin. Pharmacol. 32:361365, 1987.

56. C. Jean and R. Laplanche. Assay of isradipine and its major metabolites in biological fluids by capillary gas chromatography and chemical ion mass spectrometry. J. Chromatogr. Biomed. Appl. 428:6169, 1988.

57. G. D. Clifton, R. A. Blouin, C. DiLea, et al. The pharmacokinetics of oral isradipine in normal volunteers. J. Clin. Pharmacol. 28:3642, 1988.

58. F. L. S. Tse, J. M. Jaffe, A. E. Hassell, and H. F. Schran. Bioavailability of isradipine in young and old rats: Effect of mode of administration. J. Pharm. Pharmacol. 41:657660, 1989.

59. W. R. Hendee. Radioactive Isotopes in Biological Research. John Wiley and Sons, New York, 1973, p. 300.

60. U.S. Code of Federal Regulations, Title 21, Drugs used in research, Part 361.1. U.S. Government Printing Office, Washington, D.C., 1986, pp. 160164.

61. F. L. S. Tse, J. M. Jaffe, and J. G. Dain. Pharmacokinetics of compound 58112, a potential skeletal muscle relaxant, in man. J. Clin. Pharmacol. 24:4757, 1984.

62. W. Krause, T. H. Duka, and H. Matthes. Pharmacokinetics and biotransformation of the anxiolytic abecarnil in healthy volunteers. Xenobiotica 21:763774, 1991.

63. J. G. Wagner. Fundamentals of Clinical Pharmacokinetics. Drug Intelligence Publications, Hamilton, IL, 1975, pp. 102106.

64. C. M. Metzler, G. L. Elfring, and A. J. McEwen. A package of computer programs for pharmacokinetic modelling. Biometrics 30:562, 1974.

65. R. A. Fisher. The Design of Experiments, 8th ed. Oliver & Boyd, Edinburgh, 1966.

66. D. J. Finney. Statistical Methods in Biological Assay. Charles Griffin & Co., London, 1978.

67. E. Pitman. A note on normal correlation. Biometrica 31:912, 1939.

68. D. J. Schuirmann. A comparison of the two one-sided t-tests procedure and the power approach for assessing the equivalence of average bioavailability. J. Pharmacokinet. Biopharmaceutics. 15:657663, 1987.

69. W. J. Westlake. Symmetrical confidence intervals for bioequivalence trials. Biometrics 32:741744, 1976.

70. W. J. Dixon and F. J. Massey. Introduction to Statistical Analysis, 3rd ed. McGraw-Hill, New York, 1969.

71. N. R. Draper and H. Smith. Applied Regression Analysis, 2nd ed. John Wiley and Sons, New York, 1981, pp. 3340.

72. Fed. Reg. 43, 6968, 1978, Fed. Reg. 45:11853, 1980, Fed. Reg. 45:48160, 1980, and

Fed. Reg. 45:72200, 1980.

73. Fed. Reg. 45:56832, 1980.

74. Bioequivalence Task Force. Report by the Bioequivalence Task Force on recommendations from the bioequivalence hearing conducted by the Food and Drug Administration; Sept. 29Oct. 1, 1986. Washington, DC, January 1988.

75. A. C. Cartright, U. Gundrot-Rony, G. Rauws, et al. International harmonization and consensus DIA meeting on bioavailability and bioequivalence testing requirements and standards. Drug Inf. J. 25:471482, 1991.

76. I. J. McGilveray. Bioavailability testing of medicinal products and harmonization of international testing requirement and standards: The Canadian perspective. Drug Inf. J. 26:365369, 1992.

77. K. F. Phillips. A Log-normal model for individual bioequivalence. J. Biopharm. Stat. 3:185201, 1993.

78. Fed. Reg. 42:1648, 1977.

79. M. Eichelbaum, G. E. von Unruh, and A. Somogyi. Application of stable labelled drugs in clinical pharmacokinetic investigations. Clin. Pharmacokinet. 7:490507, 1983.

80. R. L. Wolen. The application of stable isotopes to studies of drug bioavailability and bioequivalence. J. Clin. Pharmacol. 26:419424, 1986.

81. L. M. Shaw, H. J. Bonner, L. Fields, and R. Lieberman. The use of concentration measurements of parent drug and metabolites during clinical trials. Ther. Drug Monitor. 15:483487, 1993.

82. L. P. Sananthanan and C. C. Peck. The randomized concentration-controlled trial: An evaluation of its sample size efficiency. Control. Clin. Trials 12:780794, 1991.

83. G. Levy. Variability in animal and human pharmacodynamic studies. In Variability in Drug Therapy: Description, Estimation, and Control. Edited by M. Rowland. Raven Press, New York, 1985, pp. 125138.

84. L. P. Sananthanan, C. Peck, R. Temple, R. Lieberman, and G. Pledger. Randomized PK-controlled dosing and titration: An integrated approach for designing clinical trials. Drug Inf. J. 25:425431, 1991.

85. J. W. Mandema, D. Verrotta, and L. B. Sheiner. Building population pharmacokinetic-pharmacodynamic models. I. Models for covariate effects. J. Pharmacokinet. Biopharmaceutics. 20:511528, 1992.

86. M. Davidian and A. R. Gallant. Smooth nonparametric maximum likelihood estimation for population pharmacokinetics, with application to quicidine. J. Pharmacokinet. Biopharmaceutics. 20:529555, 1992.

87. R. Laplanche, B. Fertil, E. Nüesch, J. P. Jais, W. Niederberger, and J. L. Steiner. Exploratory analysis of population pharmacokinetic data from clinical trials with application to isradipine. Clin. Pharmacol. Ther. 50:3954, 1991.

88. R. Voges, B. R. von Wartburg, and H. R. Loosli. Tritiated compounds for in vivo investigations: CAMP and 3H-NMR spectroscopy for synthesis planning and process control. Proceedings of 2nd International Symposium on Synthesis and Applications of Isotopically Labeled Compounds, Kansas City, Missouri, 1985. Elsevier Science Publishers, Amsterdam, 1986, pp. 371376.

89. B. Von Wartburg, D. Hirst. Biotransformation study in man. Document on file, Sandoz Ltd., Basel, Switzerland, 1985.

90. R. P. Hof and U. T. Rüegg. Pharmacology of the new calcium antagonist isradipine and its metabolites. Am. J. Med. 84(3B):1317, 1988.

91. R. P. Hof, A. Hof, U. T. Rüegg, N.S. Cook and A. Vogel. Stereoselectivity at the calcium channel: Different profiles of haemodynamic activity of the enantiomers of the dihydropyridine derivative PN 200-110. J. Cardiovasc. Pharmacol. 8:221226, 1986.

92. W. H. DeCamp. The FDA perspective on the development of stereoisomers. Chirality 1:26, 1989.

93. S. G. Nerurkar, S. V. Dighe, and R. L. Williams. Bioequivalence of racemic drugs. J. Clin. Pharmacol. 32:935943, 1992.

94. S. V. Dighe. Current bioavailability and bioequivalence requirements and regulations. Paper presented at the Educational Seminar sponsored by Regulatory Affairs Professional Society, August 3, 1983, Morristown, New Jersey.

95. Academy of Pharmaceutical Sciences: Report of the Ad Hoc Committee on Drug Product Selection. J. Am. Pharm. Assoc. NS 13:278, 1973.

96. A. P. Mekikian, A. B. Straughn, G. Slywka, P. L. Whyatt, and M. C. Meyer. Bioavailability of 11 phenytoin products. J. Pharmacokinet. Biopharmaceutics. 5:133146, 1977.

97. H. M. Taggart and J. M. Alderdice. Fatal cholestatic jaundice in elderly patients taking benoxaprofen. Br. Med. J. 284:1372, 1982.

98. D. G. Shand. Biological determinants of altered pharmacokinetics in the elderly. Gerontology 28(Suppl. 1):817, 1982.

99. R. Temple. Discussion paper on the testing of drugs in the elderly (memorandum). U.S. Food and Drug Administration, Department of Health and Human Services, Washington, D.C., September 30, 1983.

100. J. G. Ouslander. Drug therapy in the elderly. Ann. Intern. Med. 95:711722, 1981.

101. C. H. Kleinbloesem, P. Van Brummelen, J. Van Harten, M. Danhof, and D. D. Breimer. Nifedipine: Influence of renal function on pharmacokinetic/hemodynamic relationship. Clin. Pharmacol. Ther. 37:563574, 1985.

102. G. R. Aronoff and R. S. Sloan. Nitrendipine kinetics in normal and impaired renal function. Clin. Pharmacol. Ther. 38:212218, 1985.

103. B. Edgar, C. Regardh, G. Johnsson, L. Johansson, P. Lundborg, et al. Felodipine Kinetics in Healthy Men. Clin. Pharmacol. Ther. 38:205211, 1985.

104. M. M. Reidenberg, H. Kostenbauder, and W. Adams. Rate of drug metabolism in obese volunteers before and during starvation and in azotemic patients. Metabolism 18:209213, 1969.

105. R. Talbert. Drug dosing in renal insufficiency. J. Clin. Pharmacol. 34:99110, 1994.

106. K. Schönholzer and C. Marone. Pharmacokinetics and dialysability of isradipine in chronic haemodialysis patients. Eur. J. Clin. Pharmacol. 42:231233, 1992.

107. T. F. Blaschke and P. C. Rubin. Hepatic first-pass metabolism in liver disease. Clin. Pharmacokinet. 4:423432, 1979.

108. C. F. George. Drug kinetics and hepatic blood flow. Clin. Pharmacokinet. 4:433448, 1979.

109. D. Fabre, F. Bressolle, R. Gomeni, O. Bouvet, A. Dubois, C. Raffanel, J. Gris, and M. Galtier. Identification of patients with impaired hepatic drug metabolism using a limited sampling procedure for estimation of phenazone (antipyrine) pharmacokinetic parameters. Clin. Pharmacokinet. 24:333343, 1993.

110. J. Cotting, J. Reichen, K. Kutz, R. Laplanche, and E. Nüesch. Pharmacokinetics of isradipine in patients with chronic liver disease. Eur. J. Clin. Pharmacol. 38:599603, 1990.

111. C. H. Kleinbloesem, J. Van Haren, J. P. H. Wilson, M. Danhof, P. van Brummelen and D. D. Breimer. Nifedipine: Kinetics and hemodynamic effects in patients with liver cirrhosis after intravenous and oral administration. Clin. Pharmacol. Ther. 40:2128, 1986.

112. D. Perrier and M. Mayersohn. Noncompartmental determination of the steady-state volume of distribution for any mode of administration. J. Pharm. Sci. 71:372373, 1979.

113. L. Z. Benet and R. L. Galeazzi. Noncompartmental determination of the steady-state volume of distribution. J. Pharm. Sci. 68:10711074, 1979.

114. M. Gibaldi, R. N. Boyes and S. Feldman. Influence of first-pass effect on availability of drugs on oral administration. J. Pharm. Sci. 60:13381340, 1971.

115. L. Z. Benet. Pharmacokinetic parameters: Which are necessary to define a drug substance? Eur. J. Respir. Dis. 65:45, 1984.

116. B. P. Hamilton. Treatment of essential hypertension with PN 200-110 (isradipine). Am. J. Cardiol. 59:141B145B, 1987.

117. L. Pichard, I. Fabre, G. Fabre, J. Domergue, B. Saint Aubert, et al. Cyclosporin A drug interactions: Screening for inducers and inhibitors of cytochrome P-450 (cyclosporin A oxidase) in primary cultures of human hepatocytes and in liver microsomers. Drug Metab. Dispos. 18:595606, 1990.

118. R. D. Toothaker and P. G. Welling. The effect of food on drug bioavailability. Annu. Rev. Pharmacol. Toxicol. 20:173199, 1980.

119. K. Hirasawa, W. F. Shen, D. T. Kelly, G. Roubin, K. Tateda, and J. Shibata. Effect of food ingestion on nifedipine absorption and haemodynamic response. Eur. J. Clin. Pharmacol. 28:105107, 1985.

120. D. P. Reitberg, S. J. Love, G. T. Quercia and M. Zinny. Effect of food on nifedipine pharmacokinetics. Clin. Pharmacol. Ther. 42:7275, 1987.

121. S. S. Davis. Physiological factors in drug absorption. Ann. N.Y. Acad. Sci. 618:140149, 1991.

122. P. A. Winstanley and M. Orme. The effects of food on bioavailability. Br. J. Clin. Pharmacol. 28:621628, 1989.

123. M. Lagas and J. H. G. Joukman. Influence of food on the rate and extent of absorption of theophylline after oral single dose administration of controlled release tablet. Int. J. Clin. Pharmacol. Ther. Toxicol. 23:424426, 1985.

124. S. K. Gupta, R. C. Mantro, and S. Tomlanovich. Effect of food on the pharmacokinetics of cyclosporine in healthy subjects following oral and intravenous administration. J. Clin. Pharmacol. 30:643653, 1990.

125. R. J. Ptachcinski, R. Venkataramanan, and J. T. Rosenthal. The effect of food on cyclosporine absorption. Transplantation 40:174176, 1985.

126. B. Whiting, A. W. Kelman, and J. Grevel. Population pharmacokinetics: Theory and clinical application. Clin. Pharmacokinet. 11:387401, 1986.

127. L. B. Sheiner and L. Z. Benet. Premarketing observational studies of population pharmacokinetics of new drugs. Clin. Pharmacol. Ther. 38:481487, 1985.

128. L. P. Sananthanan and C. C. Peck. Concentration-controlled trials: Basic concepts, design, and implementation. In Integration of Pharmacokinetics, Pharmacodynamics, and Toxicokinetics in Rational Drug Development. Edited by A. Yacobi, et al. Plenum Press, New York, 1993.

129. R. J. Ptachcinski, R. Venkataramanan and G. Burckart. Clinical pharmacokinetics of cyclosporin. Clin. Pharmacokinet. 11:107132, 1986.

130. G. R. Brown, M. Miyata, and J. P. McCormack. Drug concentration monitoring: An approach to rational use. Clin. Pharmacokinet. 24:187194, 1993.

131. L. M. Shaw, L. Bowers, L. Demers, D. Freeman, T. Moyer, et al. Critical issues in cyclosporine monitoring: Report of the Task Force on Cyclosporine Monitoring. Clin. Chem. 36:18411846, 1990.

132. J. P. Reymond, J. L. Steimer and W. Niederberger. On the dose dependency of cyclosporin A absorption and disposition in healthy volunteers. J. Pharmacokinet. Biopharmaceutics. 16:331353, 1988.

133. A. van Rijthoven, B. A. Dijkmans, H. S. Gori The, et al. Cyclosporin treatment for rheumatoid arthritis: A placebo controlled, double blind, multicenter study. Ann. Rheum. Dis. 45:726731, 1986.

134. P. Tugwell, C. Bombardier, M. Gent, R. Bennett, W. Bensen, S. Carette, et al. Low dose cyclosporin versus placebo in patients with rheumatoid arthritis. Lancet 335:10511055, 1990.

14
Population Pharmacokinetics and Pharmacodynamics

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I. Introduction

Population pharmacokinetics or pharmacodynamics can be defined as the study of the variability in plasma drug concentration or pharmacological effect between individuals when standard dosage regimens are administered [1]. Nu-

merous studies of pharmacokinetics and pharmacodynamics have led to the appreciation of the great degree of variability in pharmacokinetic and pharmacodynamic parameters within a patient population. Population analysis techniques try to measure this variability within the population and account for it in terms of patient specific covariates such as age, weight, disease state, concurrent therapy, and others.

Population approaches recognize the need to model the individual in order to understand the population. The first building block of a population model is a pharmacokinetic (e.g., two-compartment) or pharmacodynamic (e.g., E_{max} model) model that characterizes the response of the system (e.g., concentration-time profile or pharmacological effect) in an individual subject. The pharmacokinetic and pharmacodynamic parameters vary from individual to individual due to variation in observable patient characteristics and variation in unobserved random effects that follow a certain multivariate distribution. The distribution of the random effects is mostly characterized by the first two moments, its mean and its variance-covariance matrix. The mean and the variance-covariance matrix completely characterize the probability distribution of the parameters if they are normally or log-normally distributed, as is often assumed in pharmacokinetic and pharmacodynamic data analysis. Population pharmacokinetics and pharmacodynamics provide three pieces of information required to identify the dosage regimens for individual patients that optimize therapeutic outcome: the population mean pharmacokinetic and pharmacodynamic parameters and their relationships with patient specific covariates; the probability distribution of interindividual random effects that is not explicable by patient-specific covariates; and the residual variability due to measurement errors and intraindividual variability. The population mean parameter values and estimated interindividual and residual variability can be used to design initial dosage regimens that will ensure that most of the patient will receive a safe and efficacious treatment [2,3]. Patient-specific covariates can be included in the model to individualize the initial dosage regimens and increase the chance of a successful treatment. If the unexplained interindividual variability is large and the therapeutic window small, Bayesian feedback approaches can be used to adjust the dosage regimen individually based on only one or several measurements obtained for that individual. Population approaches provide the necessary parameters for Bayesian dosage adjustment.

Population pharmacokinetics or pharmacodynamics is nothing new. Indeed, it has been the object of most pharmacokinetic and pharmacodynamic studies to understand the genetic, environmental, physiological, or pathophysiological factors affecting drug absorption, distribution, elimination, and effect in order to account for the large degree of interindividual variability in these processes. These studies are traditionally comparative studies in which a group of subjects with a certain characteristic likely to affect the

pharmacokinetics or pharmaco-

dynamics is compared to a group of healthy individuals. Mostly extensive measurements are taken in a small number of individuals under well-controlled conditions. The parameters of interest are calculated in each individual and standard statistical tests can be used to evaluate whether the groups are different. Probably the most important problem with these studies is that the subjects are often not a representative sample of the patient population treated with the drug.

Population approaches have recently received considerable attention [4]. This is due to the development of new analysis techniques based on nonlinear mixed-effect models that have allowed the analysis of a new source of data that is sparse in nature, namely observational data. Observational data generally consist of only a few measurements of drug concentration or effect per individual and are collected during routine clinical care of the patient or as a supplement in a study designed and carried out for another purpose. The computer program NONMEM (an acronym for nonlinear mixed-effects model), developed by Beal and Sheiner [5], was the first computer program available to analyze sparse data sets in a pharmacokinetic setting. Sheiner and colleagues have advocated the use of information gathered during routine care of patients to obtain estimates of population pharmacokinetic and pharmacodynamic parameters in the actual patient population receiving the drug. These patients may be hard to study under the rigorous conditions of well-controlled clinical trials. The application of these methods in a clinical setting has been very successful in establishing guidelines for the adjustment of dosage regimens for specific populations and optimizing individual therapy by means of Bayesian feedback algorithms [69]. The success of these applications and their potential benefits has stirred interest in the application of mixed-effects modeling approaches to the drug development program [4]. This has also led to interesting speculations on how to design phase IIV clinical trials specifically to make use of the benefits of mixed effects modeling approaches [1015]. Furthermore, mixed effects modeling approaches have gained popularity in therapeutic areas that normally generate sparse data, such as the treatment of cancer and pharmacotherapy in neonates and young children [1618].

This chapter will discuss the definitions and statistical theory of population pharmacokinetics and pharmacodynamics, review several methods and software programs to obtain estimates of population parameters, and briefly discuss its role in clinical pharmacology and drug development.

II. Model and Parameters

A pharmacostatistical model has to be specified in order to estimate the population parameters. This model describes the observations (measured responses) in two parts: an explained part and an unexplained part. The explained part is usually a function of certain

constants, called parameters, and known covariates,

called independent variables. The unexplained part is assumed to be random, with a certain probability distribution. It is further recognized that the overall variability in the measured data in a sample of individuals reflects both interindividual variation and measurement error (intraindividual variation). Throughout this chapter, y_{ij} will denote the *j*th measurement obtained in the *i*th individual. There are a total of n_i observations for each individual and a total of N individuals.

A. Individual Model

The first step of the pharmacostatistical model is to specify a model for the response of an individual within the population. In mathematical terms the *j*th measurement (e.g., plasma concentration or effect) for the *i*th individual can be related to the pharmacokinetic or pharmacodynamic parameters in the following way

$$y_{ij} = f(\phi_i, x_{ij}) + \epsilon_{ij} \quad (1)$$

where *f* is a function describing the expected value of the response for a given parameter vector f_i as a function of the covariate values x_{ij}. f_i is the vector of the model parameters for the *i*th individual and x_{ij} is the set of known covariates for this individual. The function *f* can be empirical or mechanistical and it is not the scope of this chapter to discuss the various possibilities. In pharmacokinetics exponential functions are mostly used with pharmacokinetic parameters such as clearance, volume of distribution, bioavailability, and others, whereas in pharmacodynamics mostly sigmoidal E_{max} models are used with E_{max} and EC_{50} as important pharmacodynamic parameters. The known covariates are usually such things as dose, time since drug administration, and so forth. The measured value can never be determined without errors such as assay variability, requiring the introduction of the term ϵ_{ij} to account for the (random) error between the true value and the corresponding measurement. It is important to realize that the residual variability arises from a variety of sources, such as assay variability, intraindividual variability in the model parameters within the time frame of data collection, model misspecification (incorrect pharmacokinetic or pharmacodynamic model), and errors in the recording of x_{ij} (e.g., errors in the recording of the exact time of the measurement). It is generally assumed that ϵ_{ij} are independently normally distributed with mean zero and a certain variance that may be known, postulated, or incorporated in the full model through a variance model including additional unknown parameters:

$$\epsilon_{ij} \sim N(0, \sigma^2 v(x_{ij}, \phi_i, \xi)) \quad (2)$$

Equation 2 reads as ϵ_{ij} is distributed normally with mean zero and variance $s^2v(x_{ij}, f_i, x)$. The function $s^2v(x_{ij}, f_i, x)$ is called the variance model. The variance

is assumed to be dependent upon some covariates x_{ij}, the model parameters f_i, some additional parameters x that only appear in the variance model, and a scaler s^2. From Equation 1 and 2 it follows that

$$y_{ij} \sim N(E_{ij}, C_{ij}) \quad (3)$$

where the expectation of y_{ij} is given by

$$E_{ij} = f(f_i, c_{ij}) \quad (4)$$

and the variance of y_{ij} is given by

$$C_{ij} = s^2 v(c_{ij}, f_i, x) \quad (5)$$

Estimation methods for individual and population models are often based on the statistical principle of (normal theory) maximum likelihood [19,20]. A maximum likelihood estimator chooses that set of estimates of model parameters under which the probability (likelihood) of the data is higher than it is under any alternative set. Under the distribution specified in Equation 3 the joint probability (likelihood) $l_i(y_i|f_i, x, s^2)$ of the observations for individual i given estimates of f_i, x, and s^2 is given by

$$l_i(y_i|\phi_i,\xi,\sigma^2) = (2\pi)^{-\frac{n_i}{2}} \det(C_i)^{-\frac{1}{2}} \exp\left(-\frac{1}{2}(y_i - E_i)^T C_i^{-1}(y_i - E_i)\right) \quad (6)$$

where y_i is the vector of all measurement in the *i*th individual (y_{i1}, , y_{inj}), $E_i = f(f_i, x_i)$ is the vector of model predictions for y_i, and C_i is the $n_i \times n_i$ variance-covariance matrix of y_i. For simplicity, the dependence of the measurements on the covariates is left out of the notation. According to the previous independence assumptions about ϵ_{ij}, C_i is a diagonal matrix, with diagonal elements specified by Equation 5 (j = 1, , n_i) and all off-diagonal elements equal to zero.

If enough measurements are available for a specific individual, Equation 6 can be used to obtain estimates of the model parameters for that individual. The maximum likelihood estimates of the parameters are those maximizing Equation 6. Maximizing the likelihood is equivalent to minimizing -2 times the log of the likelihood (-2LL), which is numerically more stable. The following equation represents -2LL, up to a constant term 2p

$$-2LL = \log(\det(C_i)) + (y_i - E_i)^T C_i^{-1}(y_i - E_i) \quad (7)$$

In order to estimate the model parameters by minimizing -2LL, a model for the expectation and variance of y_{ij} has to be specified. Models for the expectation are commonly known, however, one may be less familiar with variance models. The following two variance models are frequently used in pharmacokinetics and

pharmacodynamics [21], namely:

$$Cij = s2 \quad (8)$$

and

$$Cij = s2f(fi,cij)2 \quad (9)$$

Model 8 is the simplest possible variance model and is called the homoscedastic model. In this case the variance is assumed to be constant for all measurements. Model 9 is called the constant coefficient of variation (c.v.) model because it states that the coefficient of variation of the measurement yij is constant, namely s. The two models specified in Equations 8 and 9 require no additional variance parameters to be estimated. A more general version of Equation 9 is sometimes used that generalizes the homoscedastic and constant c.v. model. This model states that the variance of yij is proportional to some power, x of f(fi,xij). When the value of x is constrained to values between 0 and 2, the model compromises between the homoscedastic and constant c.v. model and thus offers greater flexibility.

The selection of a particular model for Cij should be based on the expected patterns of errors. If the range of measurements is small, as is the case in most pharmacodynamic studies, a homoscedastic error model will often suffice. However, if the range of measurements is large, which may be the case in pharmacokinetic studies in which measured concentrations can vary over several orders of magnitude, it is often more appropriate to assume a constant c.v. model. Other variance structures can easily be accommodated by transformations of the data and predictions. For example, if there is reason to believe that the ϵ_{ij} are log-normally distributed, the following model can be fitted to the data

$$\log(y_{ij}) = \log\left(f(\phi_i, x_{ij})\right) + \epsilon_{ij} \quad (10)$$

For small values of ϵ_{ij} this log-transformed model is similar to the constant c.v. model.

B. Population Model

An important part of variability in pharmacokinetic and pharmacodynamic experiments is due to differences between patients. It is generally assumed that the underlying structure of the pharmacokinetic or pharmacodynamic model is similar for each individual, whereas the actual value of the parameter vector differs from individual to individual. Mathematically this can be written as

$$fi = g(q,ci) + hi \quad (11)$$

where g is a known function that describes the expected value of fi as a function of known individual specific covariates xi, such as age, weight, disease states, and others, and the vector of population average parameters q. For simplicity it is assumed that the

covariates are constant within one individual; however, changing covariates can also be incorporated in the model by permitting the

individual pharmacokinetic or pharmacodynamic parameters to depend on j as well as i. h_i represents the random variation of the individual parameter vectors around the population prediction. It is normally assumed that the h_i are independent across individuals (i.e., $h_i h_k$ are independent for $i \neq k$). A full specification of the pharmacostatistical model requires an assumption about the probability distribution of h_i. The specific assumptions made about the probability distribution of the interindividual random effects is one of the basic differences between some of the population analysis approaches that will be discussed in more detail later. For now, we assume that h_i is a p-dimensional vector of interindividual random effects with density F. Given F, the likelihood of the observations y_i of individual i in the population can be written as

$$L_i(\Psi,F) = \int l_i(y_i|\Psi,\eta)F(\eta)d\eta \quad (12)$$

where y is the collection of all parameters (q,s^2,x) that are common to all the individuals in the population. $l_i(y_i|y,h)$ is the likelihood of the data for individual i given y and h, as calculated by equation 6. The form for $l_i(..)$ in Equation 12 differs from that given in Equation 6 because f_i has been substituted by $g(q,x_i)+h_i$ to emphasize the dependence of the density on the random effects h. For N independent individuals (i.e., h_i, h_k are independent for the $i \neq k$) the likelihood of all the data is given by

$$L(\Psi,F) = \prod_{i=1}^{N} L_i(\Psi,F) \quad (13)$$

Estimates of the population parameters y and F can then be obtained by maximizing this likelihood. However, for most pharmacokinetic and pharmacodynamic models it is difficult to calculate the integral in Equation 12 exactly because of the nonlinear dependence of the data on the random effects. As a consequence, one will not, in general, be able to derive a closed form solution to the distribution of y_i or even to the first two moments of this distribution. Several approximate methods have been proposed to simplify the calculations, which will be discussed later in this chapter.

To summarize, the following components are essential for the description of a population pharmacokinetic or pharmacodynamic model:

1. A pharmacokinetic or pharmacodynamic model that describes the expected response, (e.g., the time course of drug concentration or effect), in a specific individual

2. A model describing the relationship between patient characteristics and the pharmacokinetic or pharmacodynamic model parameters (e.g., Cl, Vd, EC_{50})

3. A population model for the interindividual random variability, which describes the unexplained (unexplained by the model specified under 2.) random variability of the model parameters in the patient population

4. A variance model for random residual variability in the data

Figure 1 gives a very simple example of a population model that clearly shows how the model predictions and random effects influence the measured response from a population point of view [20]. In this example the measured plasma concentration Cij is described by a one-compartment model parameterized in Cl and Vd according to the structure presented in Equation 1. The Cl is

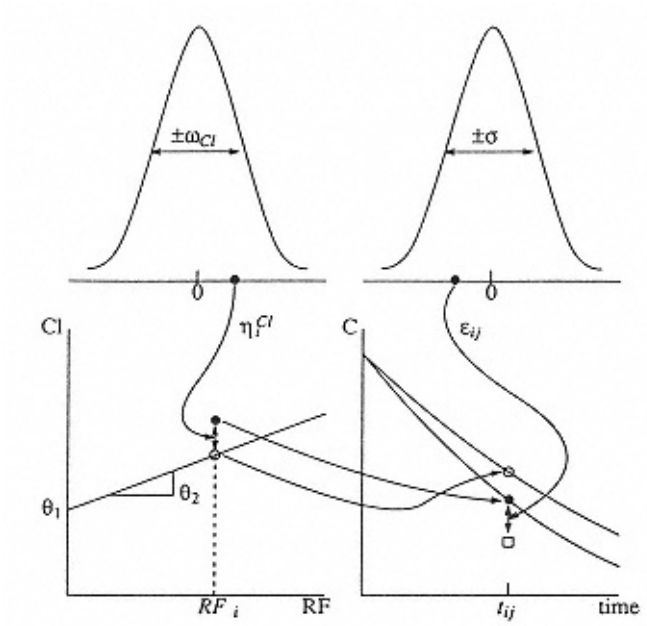


Figure 1
Influence of the random and fixed effects on the observed plasma concentrations Cij from the population point of view. The open circle, lower left, is the population mean predicted clearance (Cli) for the *i*th individual, dependent on renal function (RFi) and population mean parameters q1 and q2. The solid circle is the true clearance for the *i*th individual, which differs from the population prediction by hjC1 chosen randomly from a distribution (upper left) with mean zero and standard deviation of wcl. Lower right, the observed C at time tij (open square), differs from the true value (solid circle) by an error ϵ_{ij}, chosen independently from a distribution with mean of zero and standard deviation of s. The C corresponding to the population based prediction is also shown (upper curve, open circle). (From Reference 20.)

assumed to be linearly related to some measure of renal function (RF) according to the structure of equation 11. Interindividual variability is assumed to be present only in Cl, not in Vd. Thus, Vd is assumed to be constant in the population. ϵ_{ij} and h_i are assumed to be normally distributed with variance s^2 and w^2, respectively.

III. Methods to Obtain Population Parameter Estimates

During the past two decades a variety of methods have been proposed to estimate population characteristics of the parameters of pharmacokinetic and pharmacodynamic models. This section will give an overview of some of these approaches followed by a discussion on their performance to analyze data normally encountered in pharmacokinetic or pharmacodynamic studies. Two types of data, experimental data obtained from well-controlled experiments and observational data obtained during routine clinical care, were introduced previously as data sources to obtain pharmacokinetic and pharmacodynamic parameter estimates. The selection of a proper analysis method depends on the type of data available and also on the goals of the analysis. The performance of the presented analysis techniques will be discussed taking this into account. However, the differentiation between these two extreme sources of data may become less and less apparent since the popularity of population approaches may lead to the relaxation of design restrictions in order to increase the capacity to study the population receiving the drug [14].

A. Naive Pooled Data Analysis

It is tempting to simplify the population model presented in Equations 1 and 11 by pooling all the data together and treating the data as arising from a single individual unit. This means that all the interindividual random effects are zero, that is, all h_i in Equation 11 are zero and the *l*th measurement can be described by the following model, which is a combination of Equations 1 and 11

$$Yi = f(Q, xl) + \epsilon l \quad (14)$$

The omission of interindividual random effects simplifies the estimation problem. Under the assumption that the ϵ are independently identically normally distributed, estimates of the model parameters q and s^2 can be obtained using the same procedure as described previously for the individual's parameter and making the proper substitution for E_i and C_i in Equation 7. Relationships between pharmacokinetic or pharmacodynamic model parameters and subject specific covariates such as age, weight, and so on, can still be characterized. However, estimates of the density function F of interindividual random effects are not

obtained. This severely limits the applicability of this method if the interindividual variability in the pharmacokinetic and pharmacodynamic parameters is of interest, for example, for simulations of the spread of responses to certain dosage regimens, safety assessment, and Bayesian dosage adjustment.

The greatest advantage of the pooled analysis approach is its simplicity and the widespread familiarity of researchers with simple nonlinear regression. When the data originates from a well-balanced and controlled experiment the method can, under certain conditions, provide good estimates of the parameters that describe the population mean response [2226]. However, one has to be aware of the limitations of the approach and the problems that may occur. The model ignores the complex combination of intra- and interindividual variation implicit in the assumed population model. The interaction of these sources of variability may lead to complex and not entirely intuitive variability around the measured response that may not be correctly described by the residual error models such as presented in Equations 8 and 9 [27]. This model misspecification may result in biased estimates of q and may especially result in biased predictions for dosage regimens other than from which the model was derived. Figure 2 illustrates the influence of the source of variability on the model response as reflected in the drug concentrations. Figure 2 shows that the variability in concentrations is a complex function of when they are measured. This is a consequence of the nonlinear relationship between the model parameters and time course of drug concentrations. The figure also shows that the average concentrations at each time point do not correspond with the underlying population model. Thus, population parameters should in principle not be obtained from the average data or a pooled analysis technique that describes the average data (e.g., homoscedastic or constant c.v. residual error models). To incorporate the complex variance structure, a more flexible residual error model may be used, thereby making the approach less naive. Exceptions occur when the interindividual variability is small with respect to the other sources of variability such as measurement error and random intraindividual variation, which may often be the case in homogeneous laboratory animals but will be less so in studies involving humans. Another, more subtle, problem is that the model assumes independence of y_l, which may not be the case for two measurements that are obtained from the same physical individual.

Despite these problems, Shafer and colleagues [22,24,26] have shown that for several drugs used in anesthesia a pooled analysis approach provided population mean parameters that, when prospectively tested, accurately predicted drug concentrations after drug administration by a computer-controlled infusion pump. In all instances the data originated from well-controlled experiments with extensive sampling. Furthermore, the pooled analysis provided similar population mean parameter estimates when compared to

several other population analysis methods [23,25]. This is in contrast with an earlier simulation study that

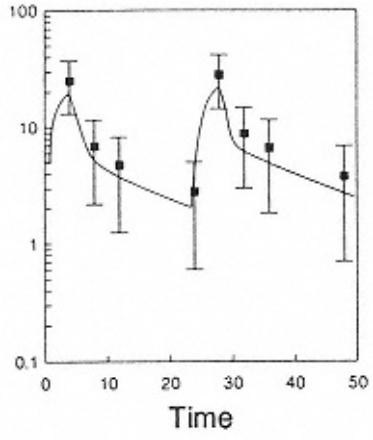


Figure 2
Simulation of the variability in observed plasma concentrations as a function of time. The simulations are based on the pharmacokinetic parameters of teniposide, an anticancer drug. For the simulation, two doses of 200 mg/m2 were given as a 4 h infusion at time 0 and 24 h. A two-compartment pharmacokinetic model was assumed. The four parameters of the two-compartment model were defined to derive from independent log-normal distributions such that the mean clearance was 0.964 L/m2/h with a coefficient of variation of 35%. The residual error model was defined to be a constant c.v. model with a coefficient of variation of 25%. The solid line represents the drug concentration time profile for the population mean pharmacokinetic parameters. The solid squares and error bars represent the observed mean concentrations and one standard deviation when 500 individuals are simulated (From Reference 27.)

showed that a pooled approach provided biased estimates of the population mean parameters even when a well-balanced experimental study design was used [28]. This discrepancy may have to do with the inappropriate weighing scheme used in the latter study or the large amount of interindividual variability present.

Another limitation does not relate to the potential bias in the parameter estimates but to the interpretation and conclusions drawn from the results. The average population response provided by the pooled analysis approach can, quite often, produce a distorted picture of the response, which is not representative for any of the individuals. An illustrative example of this is given in Figure 3. The population average response gives a false sensation of safety when one is treating the individual. The shallow slope of the concentration-effect relationship suggests a gradual increase in effect when the dose is increased, whereas the opposite is true for the individual.

A real problem with the pooled analysis approach emerges when features such as imbalance and confounding correlations are present. The two features

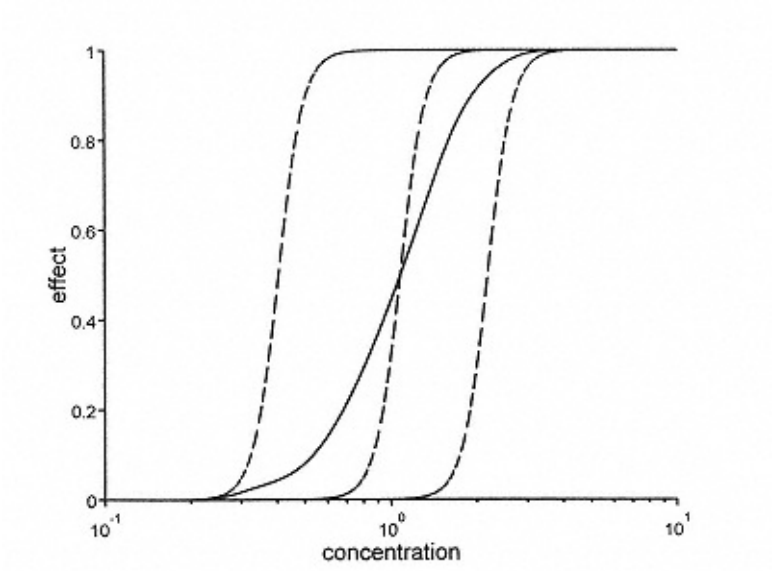


Figure 3
Comparison of individual concentration-effect relationships (dashed lines) and the population average concentration-effect relationship (solid line). The population average represents the observed mean effect of 100 simulated subjects. The sigmoid concentration-effect relationship was assumed. The EC_{50} was defined to derive from a log-normal distribution with a mean value of 1 and a coefficient of variation of 50%. The dashed lines show the concentration-effect relationships of the subjects with an EC_{50} at the 5th, 50th and 95th quantile of the simulated distribution. (Redrawn according to data from Reference 14.)

are prevalent in observational data, which makes the pooled analysis approach unsuitable for this type of data. Imbalance of the data occurs when there are many more measurements taken from one individual than the others. Consider, for example, the simple case in which 99 measurements were taken from one individual and only 1 from another. The average of all these measurements is not the best estimate of the population mean. In real data the situation may not be this extreme but may still be detrimental to the analysis.

Another problem that may occur is confounding correlations. Confounding correlations occur when the design of the study correlates with the outcome (i.e., the presence or absence of a measurement is dependent on the individual's pharmacokinetics or pharmacodynamics). Normally one would try to prevent confounding correlations by means of randomization. However, for observational data this cannot always be guaranteed. Consider a pharmacokinetic study

in which the concentrations fall below the lower limit of quantification during the study. Towards the end of the experiment only those individuals with the smallest clearance or largest volume of distribution will contribute to the measurable concentrations. This will result in a biased estimate of the terminal half-life and may be interpreted as an additional phase in the kinetic profile.

B. Two-Stage Methods

A frequently used technique to analyze experimental data is first to obtain estimates of the individual pharma cokinetic or pharmacodynamic parameters $\hat{\phi}_i$ and then combine these in some manner to characterize the distribution of these parameters (e.g., by their [population] mean and their variance-covariance matrix). This type of analysis can only be used if there are enough measurements available for each individual, such as in experimental-type data. In general, this method is unsuitable for observational data. The individual parameter estimates can be obtained by fitting an appropriate model to the data of each individual by maximum likelihood estimation as described previously. As an alternative, certain parameters, such as clearance, may be estimated using noncompartmental kinetic analysis. The distribution of the individual parameter estimates can be evaluated and appropriate summary statistics such as mean, variance, quantiles, etc., can be calculated. The population mean and variance-covariance matrix can be computed by

$$\phi = \frac{1}{N}\sum_{i=1}^{N}\hat{\phi}_i \qquad (15)$$

$$\Omega = \frac{1}{N}\sum_{i=1}^{N}(\hat{\phi}_i - \phi)(\hat{\phi}_i - \phi)^T \qquad (16)$$

where f is the estimate of the population mean vector of parameters. If there are p parameters, W is the p × p symmetrical variance-covariance matrix describing the distribution of the parameters across the population. N in Equation 16 is frequently replaced by N-p. This approach is generally referred to as the standard two-stage approach. If the parameters appear to be log-normally distributed, Equations 15 and 16 can be used after logarithmic transformation of the individual parameter estimates. Relationships between patients' characteristics and the estimated pharmacokinetic or pharmacodynamic parameters can easily be established by linear or nonlinear regression analysis. A disadvantage of the two-stage approach is that it does not provide standard errors for estimates of the variance-covariance matrix.

There are some subtle problems associated with the calculation of the population mean and variance-covariance matrix according to Equations 15 and

16. The individual parameters used to calculate the mean and covariance matrix are estimates of the individual's parameters obtained by nonlinear regression analysis and not the true parameters. The degree of imprecision in the parameter estimates can be quite large, especially in the case of poor experimental design, a small number of measurements, or a high level of the measurement error (large s^2). Furthermore, the individual parameters will not all be estimated with the same precision. Because of this the two-stage estimate of f is possibly biased. Consider the previous extreme example in which 99 measurements were obtained from one individual and only one from another. The mean of the two individual averages is in this case an obvious bad estimate of the population mean parameters. A more serious problem is encountered with the two-stage estimate of W. The estimate of W will be upwardly biased because it includes both interindividual variability and variability due to imprecision in the estimates of f_i. If the imprecision of the parameter estimates is small with respect to the interindividual variability, this bias may not be too serious. Simulations have confirmed the predictions from statistical theory. The standard two-stage approach provides upwardly biased estimates of W [28,29] and biased estimates of both f and W if the number of data points per individual decreases or residual error increases [30,31]. Several improvements to the standard two-stage approach have been proposed to obtain less biased estimates of the population parameters [29,32] and will be discussed next.

1. Global Two-Stage Approach.

The global two-stage approach acknowledges that the individual parameter estimates are imprecise. The asymptotic covariance matrix of $\hat{\phi}_i$ deenoted as V_i, is a measure of the imprecision in the parameter estimates and is used in the calculations. Most nonlinear regression programs provide an estimate of V_i (see, e.g., [33]). An extensive description of the method is provided by Steimer et al. [29]. The global two-stage approach provides a maximum likelihood estimate of f and W by an iterative method. The method assumes that the estimates of the individual parameters are normally distributed around the true parameters, with variance V_i. The contribution of each individual's parameter estimate to the population parameters is adjusted for the accuracy of those estimates. The *l*th step of the global two-stage procedure first provides refined estimates of the individual parameters f_i

$$\phi_i^{(l)} = (V_i^{-1} + \Omega^{(l-1)-1})^{-1}(V_i^{-1}\,\hat{\phi}_i + \Omega^{(l-1)-1}\,\phi^{(l-1)}) \qquad (17)$$

where $f_i(k)$, $f(k)$ and $W(k)$ denote the estimates of f_i, f, and W at the *k*th iteration. $\hat{\phi}_i$ and V_i are the individual parameter estimates and asymptotic covariance matrix obtained from the individual fits. Then refined estimates of the population

parameters are obtained

$$\phi^{(l)} = \frac{1}{N}\sum_{i=1}^{N}\phi_i^{(l)} \quad (18)$$

$$\Omega^{(l)} = \frac{1}{N}\sum_{i=1}^{N}(\phi_i^{(l)} - \phi^{(l)})(\phi_i^{(l)} - \phi^{(l)})^T + \frac{1}{N}\sum_{i=1}^{N}(V_i^{-1} + \Omega^{(l-1)-1})^{-1} \quad (19)$$

The first term of W is the maximum likelihood estimate of W assuming $f_i^{(1)}$ to be the known value of f_i (compare Equation 16). The second term accounts for the fact that $f_i^{(1)}$ is only an estimate of f_i.

Simulations have shown that the global two-stage approach provides unbiased estimates of the population mean parameters and their variance-covariances, whereas the estimates of the variances were upwardly biased if the standard two-stage approach was used [29]. However, the simulations were done under the ideal situation that the residual error is gaussian with a known variance. It is well known that the asymptotic covariance matrix V_i used in the calculations is approximate and that the approximation can be poor under less ideal conditions [34,35].

2. Bayesian Two-Stage Approach

An alternative method that is Bayesian in nature has been proposed by Racine-Poon [32]. This method uses the estimates of the individual parameters $\hat{\phi}_i$ and asymptotic covariance matrix V_i obtained from the individual fits, with very weak assumptions about the prior distribution of the population parameters to calculate a posterior density function from which f and W can be obtained. An iterative method suggested by Dempster et al. [36], the so-called EM algorithm, is used to calculate the posterior density function. At the *l*th iteration first improved estimates of f are calculated

$$\phi^{(l)} = D^{(l)}\sum_{i=1}^{N}(V_i + \Omega^{(l-1)})^{-1}\hat{\phi}_i \quad (20)$$

where

$$D^{(l)} = \left(\sum_{i=1}^{N}(V_i + \Omega^{(l-1)})^{-1}\right)^{-1} \quad (21)$$

Conditioning on $f = f^{(1)}$ and $W = W^{(1-1)}$, improved estimates of $f_i^{(1)}$ are obtained

$$\phi_i^{(l)} = (V_i^{-1} + \Omega^{(l-1)-1})^{-1}(V_i^{-1}\hat{\phi}_i + \Omega^{(l-1)-1}\phi^{(l)}) \quad (22)$$

Conditioning on $f_i = f_i(1)$, the estimate of W is improved

$$\Omega^{(l)} = \frac{R + \sum_{i=1}^{N} (\phi_i^{(l)} - \phi^{(l)})(\phi_i^{(l)} - \phi^{(l)})^T}{N - p} \quad (23)$$

where R reflects the prior information about the population variance-covariance matrix. R can be taken as dI, where I is the identity matrix of dimension p with d being small enough so as not to influence the posterior estimate [37]. The iterations are continued until W converges.

Simulation studies have shown that the Bayesian two-stage approach provides good estimates of the population parameters under several practical conditions [32,38]. However, the application of this method to pharmacokinetic or pharmacodynamic data analysis has still been limited [3740].

C. Nonlinear Mixed-Effects Modeling

Nonlinear mixed-effects modeling approaches were originally introduced to characterize the pharmacokinetics or pharmacodynamics in the actual patient population treated with the drug by making use of sparse data (e.g., a few samples from each individual collected during routine patient management). The underlying idea was that some of these patients, especially the elderly, critically ill, and children, cannot be exposed to the rigors of a well-controlled clinical trial with extensive blood sampling and effect measurements and are therefore not well-represented in the pharmacokinetic and pharmacodynamic studies required for drug approval. The number of measurements per patient is typically small, ranging from one to five. This precludes the use of traditional two-stage analysis techniques because there are not enough data to estimate the pharmacokinetic or pharmacodynamic parameters for each individual separately. The model may be unidentifiable in a specific individual or there are too few measurements to estimate the parameters accurately.

Nonlinear mixed-effects modeling approaches analyze the data of all individuals at once, as does the pooled analysis technique, but take the interindividual random effects structure into account. This ensures that imbalance and confounding correlations that may occur in observational data are properly accounted for. Most of the approaches estimate the parameters by means of maximum likelihood. The parameters that maximize the likelihood of the population data as described by Equation 13 are selected. For most pharmacokinetic and pharmacodynamic models it is difficult to calculate the likelihood of the data, because of the nonlinear dependence of the observations on the random parameters h_i and possibly ϵ_{ij}. Several approximate methods have been proposed to deal

with this problem and will be discussed next. Apart from the approximation

used, these methods differ widely in their representation of the probability distribution of the interindividual random effects.

1. NONMEM and Other Linearization Approaches

The NONMEM program developed by Sheiner and Beal [7] was the first nonlinear mixed effects modeling approach introduced for pharmacokinetic and pharmacodynamic data analysis. NONMEM linearizes the model in the random effects by using a first-order Taylor series expansion with respect to the random variables hi and ϵ_{ij}. NONMEM is not the only method that uses this type of linear approximation. Other methods have been described by Lindstrom and Bates [41] and Vonesh and Carter [42], but have not been applied extensively to pharmacokinetic or pharmacodynamic data analysis.

A general model for the *j*th measurement in the *i*th individual of the population can be obtained by substituting the model for the individual's parameters (Equation 11) into that for the model for observations on the individual (Equation 1)

$$y_{ij} = f(\theta, x_{ij}, \eta_i) + \epsilon_{ij} \quad (24)$$

The first-order Taylor series expansion of this model with respect to the random variables hi and ϵ_{ij} around zero is given by

$$y_{ij} = f(\theta, x_{ij}) + G_{ij}(\theta, x_{ij})\eta_i + \epsilon_{ij} \quad (25)$$

where

$$G_{ij}(\theta, x_{ij}) = \left.\frac{\partial f(\theta, x_{ij}, \eta_i, \epsilon_{ij})}{\partial \eta_i^T}\right|_{\eta_i = 0} \quad (26)$$

Gij(q,xij) is the 1 × p matrix of first derivatives of *f*(qx*i*, hiϵ,) with respect to hi, evaluated at hi equals zero. In this example the model is linear in ϵ_{ij} and no approximation has to be made with respect to ϵ_{ij}. Linearity in ϵ_{ij} can often be ensured by an appropriate transformation of the data (e.g., logarithmic). The NONMEM approach further assumes that the random effect parameters ϵ_{ij} and hi are independently (multivariate) normally distributed with mean and variance given by

$$f(\theta, x_{ij}, \epsilon_{ij}, \eta_i) \quad (27)$$

$$\epsilon_{ij} \sim N(0, \sigma^2) \quad (28)$$

where W is the p × p covariance matrix of the p vector hi. Based on the distributional assumptions of ϵ_{ij} ij and hij are independent, and the linearization described by Equation 25, the expectation and variance-covariance of all observations for individual i (first two

moments) are given by

$$E_i = f(q, c_i) \quad (29)$$

and

$$Ci = Gi(Q,xi)_{WGi}(Q,xi)T + s2Ini \quad (30)$$

$f(q,xi)$ is the vector of model predictions of yi, Gi(q,xi) represents the ni × p matrix of first derivatives of $f(Q,xij,hi,\epsilon i)$ with respect to hi, evaluated at hi equals zero, and Ini represents the identity matrix of size ni. Maximum likelihood estimates of the population parameters q, W and s2 can be obtained by minimizing -2 times the log of the population likelihood given by Equation 13

$$-2LL = \sum_{i=1}^{N} (\log(\det(C_i)) + (y_i - E_i)^T C_i^{-1}(y_i - E_i)) \quad (31)$$

The approach described above is called the first order (FO) method in NONMEM. To date, the FO method is probably the most widely used approach for population pharmacokinetic and pharmacodynamic analysis. It has been evaluated by simulation [28,30,31,35,43] and used extensively for pharmacokinetic data analysis (for a recent review see [20]) and, to a lesser extent, for pharmacodynamic data analysis [37,4449]. The greatest limitation of the FO approach is that it uses a first-order Taylor series expansion to approximate the nonlinear model in hi and possibly ϵ_{ij} by a linear model in these parameters. Because of this approximation, the performance of the FO approach in terms of bias and precision of the population parameter estimates has to be evaluated by simulation. Sheiner and Beal have evaluated the performance of the FO approach for the analysis of observational data from a Michaelis-Menten pharmacokinetic model [43], observational data arising from a monoexponential pharmacokinetic model [30,31], and experimental data arising from a two-compartment model [28]. In all instances a comparison was made with the naive pooled data approach and standard two-stage approach. Overall, the FO approach outperformed the pooled data approach and standard two-stage approach for the analysis of both types of data. Figure 4 shows the performance of the standard two-stage and FO approach when data were simulated according to a simple one-compartment model with two plasma concentrations measured in each of the 50 individuals. When there is no residual error in the measurements (left panel), the estimates of clearance and volume are exact and the standard two-stage approach is known to provide good estimates of the population mean parameters and their variances. It is interesting that the FO approach also provides good estimates, despite the approximation. If the residual error increases the standard two-stage approach quickly deteriorates, especially with respect to the variance parameters. The FO approach, however, still performs reasonably well but the bias and imprecision of the estimates seems to increase with increasing residual error. The good performance of the FO approach for sparse data analysis has also been confirmed by a series of studies that

randomly de-

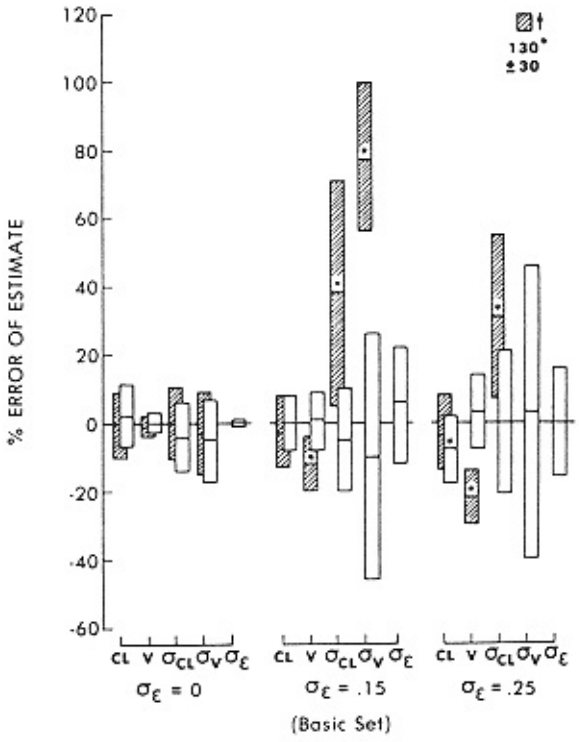


Figure 4
Bias and precision of population parameter estimates by the standard two-stage method (shaded bars) and FO method (open bars). Observational pharmacokinetic data were simulated according to a simple one-compartment pharmacokinetic model with repetitive bolus dosing. One sample is composed of 50 individuals with two drug concentrations measured in each. A Monte Carlo simulation involving 30 replications of each data set was performed. The ordinate is in units of % error (%E), defined as (estimate/true value - 1) × 100. The average %E over the 30 replications measures the bias in the estimator. It is indicated by the horizontal line in the middle of the bar corresponding to the parameter and the method. Significant ($p < 0.05$) biases are indicated by asterisks in the bars. The full height of the bar is two standard deviations of the %E. This measures the precision of the estimator. The distribution of the pharmacokinetic parameters and residual variability is assumed log-normal. The parameters are Cl, clearance; V, volume of distribution; s_{Cl}, approximate interindividual coefficient of variation of V; s_ϵ approximate coefficient of variation of residual error. The three panels show the results of the simulations for each population parameter for both methods according to the described design with increasing residual

error magnitudes s_ϵ = 0% 15%, and 25%. (From Reference 30.)

leted observations from a data-rich set and compared the parameter estimates from the full and sparse data set [13,16,44,50,51]. The good correspondence between parameter estimates obtained in these two situations suggests that the FO approach can be used to estimate population parameters using only a few measurements per individual. This is especially ideal in those individuals in whom multiple samples are difficult to obtain (e.g., blood samples for plasma concentration determination in neonates or young children).

As expected, for experimental data both the standard two-stage and FO approach estimate the population mean parameters equally well, but the FO approach provides superior estimates of the interindividual random effect parameters [28]. The variance parameters are relatively unbiased but not estimated very precisely. This problem has been attributed to the small number of individuals normally present in experimental type of studies, because the estimates of variability are considerably less precise, given a small number of individuals, than are estimates of the means. With the FO approach, the bias tends to decrease and the precision tends to increase as the number of individuals increases [30].

Beal [31] evaluated the impact of the linearization approximation of the FO approach for a simple one-compartment model by comparing the performance of this approach with the exact solution to the population likelihood. For this simple model no difference in performance was observed, which indicates that the approximation used in the FO is not detrimental to the analysis under the conditions evaluated (interindividual variances were 25%). However, other simulation studies showed that the FO approach has a potential for providing modestly biased estimates [28,29,35,38]. White et al. [35] showed that for a one-compartment multidose situation biased estimates are more likely when residual variability and interindividual variability are very high. The observed bias may be caused by the fact that the first-order Taylor series expansion is not a particularly good approximation of the underlying real (log-normal) distribution used to generate the simulated data in both studies. Another reason may be that the first-order Taylor series expansion is evaluated at η_i equals zero (the population mean estimate of η_i). Depending on the magnitude of the interindividual variability and the nonlinearity of the model, this may not be a good approximation. In analyzing real data this can be partly compensated for by including explanatory covariates in the model to reduce the variance of η_i. Steimer et al. [29] showed that for a one-compartment experimental data set the global two-stage approach outperformed the FO approach with respect to bias and precision of the both the population mean and variance estimates. Similar results were obtained by Racine et al. [38] when comparing the FO approach and the Bayesian two-stage approach.

An improved approximation recently became available in the NONMEM program [52]. The population likelihood as described by Equation 9 is calculated using a general

approximation approach to integrals attributable to the mathe-

matician Laplace, and described by de Bruijn [53]. The approximation involves first and second derivatives of$2\log(l_i(y_i|x_\epsilon))$with respect to h evaluated at some estimate of h (for a complete mathematical description see [52]). In general, this approximation can produce reasonable results as long as the posterior distribution is dominated by a single mode. In contrast to the FO approach, the approximation is evaluated at the conditional estimate of h_i and not the population mean of h_i:zero. It is beyond the scope of this chapter to describe the full details of the approximation; however, the flavor of the two new estimation methods that are available in the latest version of NONMEM will be described. One of the methods, called the laplacian estimation method uses the full Laplacian approximation, including first and second derivatives, to the likelihood as described above. The other method, called the first order conditional estimate (FOCE) method approximates the second derivative terms using the first derivatives, thereby eliminating the need to calculate the second derivatives. When a homoscedastic intraindividual error model is specified, the approximation used in the FOCE method is similar to the approximation proposed by Lindstrom and Bates [41]. However, the FOCE method is not limited to a homoscedastic intraindividual error model.

Under the model and distribution assumptions specified by equations 2428, the FOCE approach basically evaluates the first derivative terms $G(q,x_{ij})h_i$ in equation 25 at the conditional estimate of h_i. Under this model, the expectation of y_i is given by

$$E_i = f(\theta, x_i, \hat{\eta}_i) - G_i(\theta, x_i, \hat{\eta}_i)\hat{\eta}_i \quad (32)$$

where $\hat{\eta}_i$ is the estimate of h_i conditional on the current estimates of q, W, and s^2. $G(q,x_i,\hat{\eta}_i)$ represents the $n_i \times p$ matrix of first derivatives of $f(q,x_i,\hat{\eta}_i,\epsilon_i)$ with respect to h_i, evaluated at $\hat{\eta}_i$. The variance of y_i can be described by Equation 30, however the first derivative terms $G_i(q,x_i)$ are evaluated at $\hat{\eta}_i$ similar as in Equation 32. These new terms for E_i and C_i can be substituted in Equation 31 to obtain maximum likelihood estimates of the population parameters q, W and s^2.

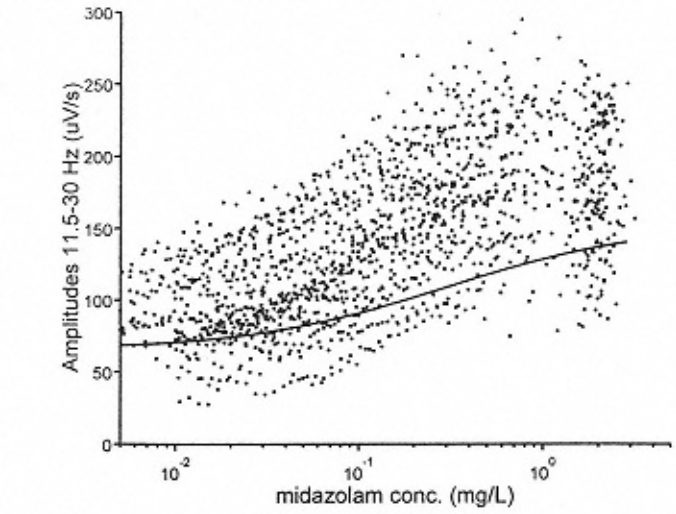
The FOCE and laplacian estimation methods are difficult and computationally intensive, because for each set of population parameter estimates conditional estimates of h have to be obtained for each individual. Thus the method involves multiple minimizations within a minimization. Depending on the complexity of the second derivatives, the laplacian method can be considerably more computationally expensive than the FOCE method. An estimate of h_i is obtained by maximizing the empirical Bayes posterior density of h_i, given the measurements y_i for that individual and conditional on the present estimates of the population parameters. For the example of the homoscedastic error model, empirical Bayes of h_i are computed as the values $\hat{\eta}_i$ that minimize the following Bayesian ob-

jective function

$$\log(\det(\sigma^2 I_{n_i})) + (y_i - f(\theta, x_i, \hat{\eta}_i))^T(\sigma^2 I_{n_i})^{-1}(y_i - f(\theta, x_i, \hat{\eta}_i)) + \hat{\eta}_i^T \Omega^{-1} \hat{\eta}_i$$

The Bayesian objective function balances the uncertainty in the individual's parameters hi against the uncertainty in the measured response. This ensures that even with few data per individual unique estimates of hi can be obtained. From the conditional estimates of hi, empirical Bayes estimates of an individual's pharmacokinetic or pharmacodynamic parameters fi can be obtained by evaluating Equation 11. These parameters can be used for Bayesian feedback adjustment of individual dosage regimens, which is one of the main applications of population parameters. The empirical Bayes estimates of the individual's model parameters are also very useful to find efficiently the relationships between the pharmacokinetic or pharmacodynamic parameters and patient specific covariates [54,55]. Both graphic [54] and automatic [55] procedures have been described to discover these relationships. The FOCE and laplacian method are still relatively new and their performance has not been evaluated extensively by simulation or application to real data sets. From a theoretical point of view the FOCE and laplacian methods should be able to correct some of the bias in the parameter estimates resulting from the FO approach. The greatest disadvantage of the FOCE and laplacian methods is that they are considerably more computationally expensive than the FO approach. The question of when to use these improved approximations instead of the FO approach remains. A descriptive overview is given by Beal and Sheiner [52]. Sometimes the bias of the FO approach is obvious because of a clear bias in the fit that is not due to structural model misspecification. Figure 5 gives an example of a clear bias resulting from the FO method (top panel). The data in this example are of a pharmacodynamic nature and characterized by the highly nonlinear sigmoid E_{max} model. A high interindividual variability in EC_{50} is also present. The bias is resolved when the FOCE method is used (bottom panel). Often the bias may not be that easily detected, especially when there is a limited number of measurements from each individual. In those situations the FOCE or laplacian method may be used to validate the final population model derived with the FO method. In principle, the FOCE method should not perform worse than the FO method and the laplacian method should not perform worse than the FOCE method. A change in parameter estimates may indicate that the FO approach should be abandoned in favor of one of the others. In some situations it can be anticipated that the FO method may perform badly. This will occur when the model is highly nonlinear in the random effects, such as the sigmoid E_{max} pharmacodynamic model or the models used to describe categorical response data, or when the interindividual variability in the model parameters is large. The multiexponential pharmacokinetic models are reasonably linear in the random effects, which is probably why the FO approach has performed so well in

population pharmacokinetics.



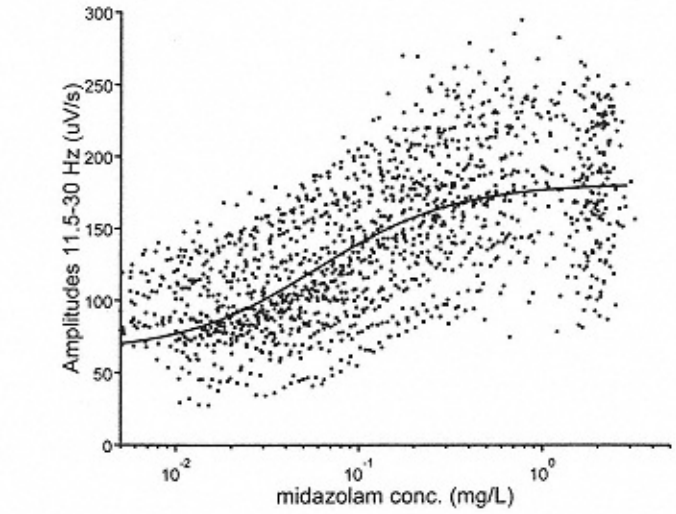


Figure 5
Plasma concentration vs. EEG effect relationship (amplitudes in the 11.530 Hz frequency band) for the benzodiazepine midazolam in the rat. The measurements were obtained after a 15 min intravenous infusion of 5 mg/kg from a total of 38 rats. The top panel shows the fit of the sigmoidal Emax model (solid line) to the data (solid dots) using the FO approach with the computer program NONMEM. The bottom panel shows the fit of the sigmoidal Emax model (solid line) to the data (solid dots) using the FOCE approach.

The NONMEM method assumes that the intra- and interindividual random effect parameters ϵ_{ij} and h_i are (multivariately) normally distributed. This does not restrict the approach to the assumption that the model parameters f_i or the residuals $y_{ij} - f(f_i, x_{ij})$ are normally distributed. Other distributions can be evaluated by specific transformations of ϵ_{ij} and h_i. An exponential interindividual error model is often used in pharmacokinetic and pharmacodynamic data analysis because it has several attractive features.

$$f_i = q \exp(h_i) \quad (34)$$

This model will ensure that the pharmacokinetic parameters are strictly positive. This is especially important when empirical Bayes estimates of h_i are obtained during the minimization. The exponential error model will prevent the occurrence of nonphysiological negative valued clearances and volumes. Another important consideration is that skewed distributions of pharmacokinetic and pharmacodynamic parameters are often observed, which may be better represented by an exponential error model. Last, the elements of W become dimensionless and express approximate coefficients of variation in the model parameters [21]. However, the actual results of specific transformations such as specified by Equation 34 are dependent on the approximation method used in NONMEM. For example, the exponential interindividual error model specified in Equation 34 is similar to the constant c.v. model when the FO method is used and will give different results when the FOCE method is used. On the other hand, an exponential residual error model will provide the same results as the constant c.v. model under both the FO and FOCE approximation. Linearization approximations in ϵ_{ij}, however, can be prevented by appropriate transformations of the predictions and observations, in this case, logarithmic.

The NONMEM method has been criticized for making a specific parametric, mostly normal or log-normal, assumption regarding the distribution of the random effects and for estimating only the first two moments of the distribution. Departures from normality such as multimodality or excess dispersion will not be detected under these assumptions. Misspecification of the distribution of the random effects may, for instance, influence the Bayesian estimation of individual parameters from new measurements for a new individual used for dosage adjustment. Bayesian forecasting is sensitive to the form of the distribution of the interindividual random effects for the calculation of individualized parameter values [3,56,57]. Multimodal or skewed distributions of the pharmacokinetic and pharmacodynamic parameters can be found if a relevant patient specific covariate such as age or genetic difference is omitted from the description of the population model. The initial work of Mallet [58] and co-workers [59] and later Davidian and Gallant [60] has demonstrated the importance of estimating the entire probability distribution F of the

interindividual random effects. The non-parametric maximum likelihood (NPML) approach introduced by Mallet [58]

provides a nonparametric discrete estimate of F. The seminonparametric (SNP) method introduced in the pharmacokinetics literature by Davidian and Gallant [60] produces a smooth nonparametric estimate of the entire random effects density. The basic principles of these approaches will be discussed next.

2. Nonparametric Maximum Likelihood

The basic feature of the NPML approach introduced by Mallet [58] is that it provides an estimate of the whole probability distribution of the pharmacokinetic and pharmacodynamic parameters on a nonparametric basis. The method relies on maximization of the likelihood of the set of observation of all individuals to estimate the distribution of the parameters. Thus, the basic conceptual framework remains the same as described in above. The difference is that no specific model for the relationship between pharmacokinetic or pharmacodynamic parameters and patient specific covariates is specified. The individual parameters f_i are assumed to be independent realizations of a given random variable j with probability distribution F(f). The likelihood of all the data is given by

$$L(F) = \prod_{i=1}^{N} \int_{D} l_i(y_i|\phi) F(\phi) d\phi \quad (35)$$

where $l_i(y_i|f)$ is the likelihood of the observations y_i for the *i*the individual given f. D is the domain in which the parameters lie. Maximization of this likelihood provides an estimate F of the probability distribution of the parameters. Mallet [58] proved that this distribution is discrete, involving Np locations, where Np is less than or equal to N (the number of individuals). A specific algorithm was developed to estimate the Np locations q_k and their corresponding frequencies a_k. The number of locations depends on the level of residual error and how well the parameters are known for each individual. If the parameters are known very precisely for all N subjects, there will be N locations, each with a frequency of 1/N. The set of locations q_k and frequencies a_k completely specifies the estimate of the distribution of the parameters

$$\hat{F} = \sum_{k=1}^{N_p} \alpha_k \cdot \delta(q_k) \quad (36)$$

where d(x) denotes the Dirac probability distribution, which takes the value 1 at x and 0 elsewhere. This method provides a complete description of F with only very soft assumptions, namely that F takes only positive values and that its integral over the D domain is equal to unity [58,59]. Figure 6 gives an example of the NPML estimate of the distribution of clearance and volume of distribution obtained from data simulated according to a one-compartment model with a bimodal distribution for clearance. For

both parameters the NPML estimate describes the distribution accurately, even though only one measurement

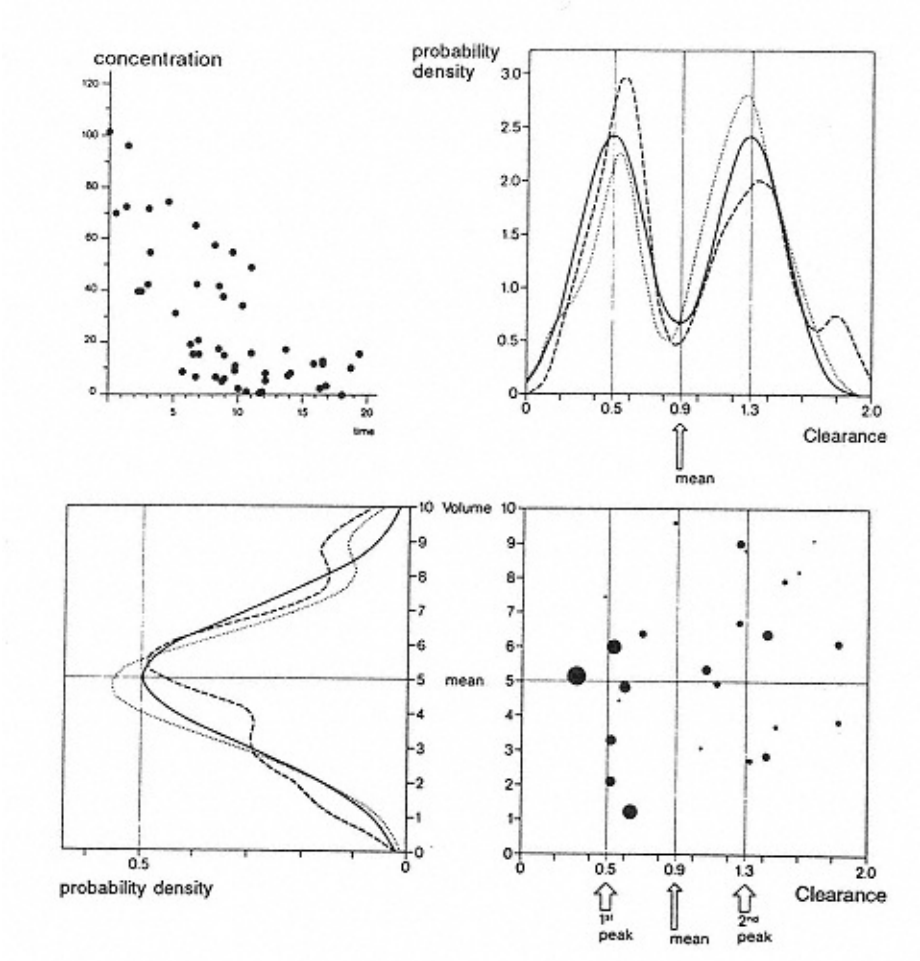


Figure 6

NPML estimate of the distribution of the pharmacokinetic parameters clearance and volume of distribution. The upper left panel shows the simulated data generated according to a one-compartment pharmacokinetic model with bolus input. A normal distribution was assumed for the volume of distribution (mean 5 and standard deviation 2) and a bimodal distribution (equal mixture of two normal distributions with respective means 0.5 and 1.3 and standard deviation 0.2) was assumed for clearance. The solid dots represent 50 time-concentration measurements, one for each individual. The lower right panel shows the estimated discrete distribution displayed as full circles. The circles are centered on the estimated locations, with radius proportional to the corresponding estimated frequency. Note that the number of estimated locations (np = 25) is less than the number of individuals in the sample (n = 50). The upper right and lower left panels display the theoretical (solid line), simulated (dotted line), and estimated (dashed line) marginal distributions for clearance and volume of distribution, respectively, after smoothing of the latter two probability distributions. (From Reference 59.)

was available per individual. If necessary, several summary statistics such as the mean or variance-covariance matrix can easily be calculated from the distribution of F specified by Equation 36.

Patient-specific covariates can also be included without specifying an a priori relationship between the pharmacokinetic or pharmacodynamic parameters and covariates. The covariates are treated as additional parameters and the algorithm provides an estimate of the joint distribution of the pharmacokinetic-pharmacodynamic parameters and the covariates [2]. From this distribution, the probability distribution of the parameters conditional on any value of the covariates can be computed and used for initial dosage selection. This also allows one to explore nonparametrically the shape of the relationship between parameters and covariates.

The main limitation of the NPML approach seems to be that the residual error model has to be known. Thus, the method is nonparametric with respect to the interindividual random effects but requires the intraindividual error to be specified a priori. In the pharmacokinetic data sets that have been analyzed with the NPML approach the residual error model was based on variance of the assay to determine the drug concentrations [2,57,61,62]. This does not seem to be realistic. Often, model misspecification and intraindividual variability will contribute considerably to the residual error. Particularly if the data is collected during several routine clinical visits spread out over several weeks, the day-to-day variability in an individual's parameters is expected to contribute to the residual error. The sensitivity of the approach to misspecification of the residual error model is presently unknown and should be evaluated.

Another limitation is that the estimator of the distribution is a point estimator and no results on the accuracy of the estimation are obtained. Thus, care should be taken in interpreting the results, especially when they are obtained from a small sample size. This may not be as much of a problem, however, if the NPML approach is primarily used for exploratory analysis and to improve the efficiency of subsequent parametric analysis. Finally, the NPML approach is a computationally expensive approach, which may limit the practicality of the approach when the dimension of the parameter space increases (e.g., in those instances in which there is a complex pharmacokinetic or pharmacodynamic model and numerous covariates).

The experience with the NPML approach in pharmacokinetic and pharmacodynamic data analysis is limited. A few simulations have shown that the method can accurately identify the distribution of the model parameters under several distributional assumptions [58]. The approach has been applied to the pharmacokinetic analysis of gentamicin [57], cyclosporin [61], and zidovudine [62]. However, the lack of generally available software

has probably limited a more extensive evaluation of this approach. Figure 7 shows the distributions of the clearance and initial volume of distribution of cyclosporin estimated using

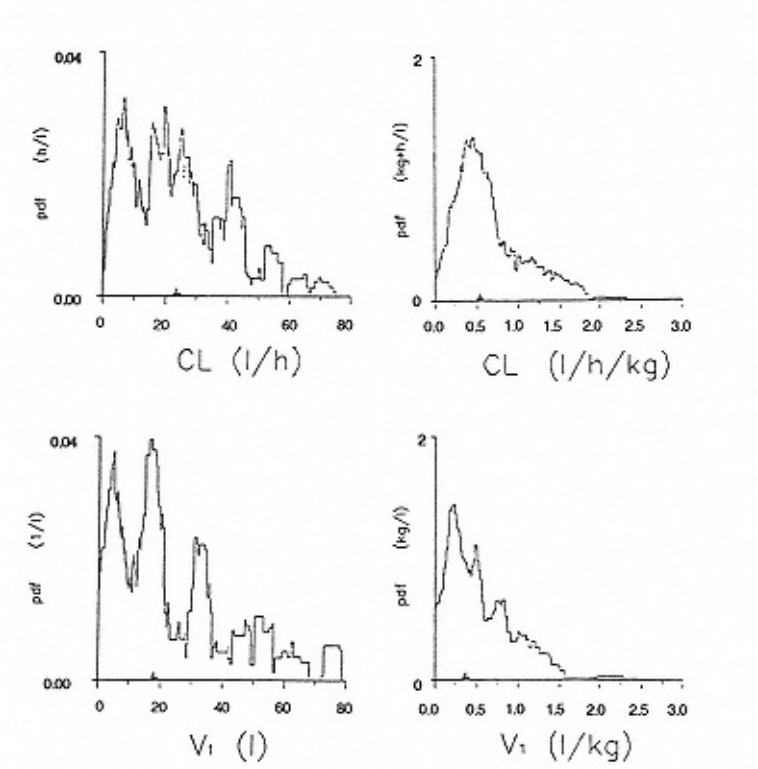


Figure 7
NPML estimate of the distribution of the pharmacokinetic parameters clearance (CL) and initial volume of distribution (V1) of cyclosporin. A two-compartment model was fitted to cyclosporin plasma concentration measurements (total of 1487 samples) obtained from 188 bone marrow recipients previous to transplantation, after intravenous infusion of the drug. The left panels show the smoothed NPML estimated distributions of CL and V1. The right panels show the smoothed NPML estimated distributions of body-weight-normalized Cl and V1. (From Reference 61.)

the NPML approach. This example shows that the distributions are not adequately approximated by a simple parametric normal or log-normal distribution. Although the several modes of the estimated distribution of clearance and initial volume of distribution no longer appear when the parameters are weight-normalized, weight is a significant covariate in the model. Similar results have been obtained with the other two drugs, indicating the importance of relaxing the assumptions on the distribution of the interindividual random effects.

3. Seminonparametric Maximum Likelihood

The seminonparametric (SNP) maximum likelihood approach originating from the field of economics was introduced in the field of pharmacokinetics by Davidian and Gallant [60,63]. Similar to the previous method, the SNP approach provides an estimate of the entire distribution of the interindividual random effects. However, instead of maximizing the likelihood over all distribution functions, the SNP method maximizes the likelihood over a class of distributions restricted to have a smooth density. This smoothness assumption is flexible enough to allow heavy-tailed, multimodal, and skewed distributions to be characterized, but prevents kinks, jumps, and oscillatory behavior [63]. This method also relies on maximization of the likelihood of the set of observation of all individuals to estimate the distribution of the random effects. Thus, the basic conceptual framework remains the same as described above and the likelihood of the data can be described by Equations 12 and 13. However, the representation of the probability distribution and calculation of the likelihood are different from the NONMEM and NPML approach. Gallant and Nychka [64] showed that the smooth distribution can be presented as an infinite series expansion, and they provide a full mathematical description. An approximation of the infinite expansion to a finite number of leading terms is used in the SNP approach. The number of terms retained is determined by a single tuning parameter. If the value of this tuning parameter equals zero, the density is (multivariate) normal. The larger the value of the tuning parameter, the more flexible the distribution. Selection of an appropriate value of this parameter is an important step in the modeling procedure [60]. The density of the random effect parameters is represented by a multivariate normal distribution multiplied by a polynomial. Another useful feature of the SNP approach is that it computes the integral present in the population likelihood by quadrature. Thus, there is no linearization approximation to the likelihood, as is used in the NONMEM approach. An advantage over the NPML approach is that standard errors can be computed for the model parameters and used for inference.

Figure 8 shows an example of the approach when applied to data simulated from a one-compartment pharmacokinetic model with a bimodal distribution in clearance. This figure shows that the method can accurately characterize the true distribution of the parameters. A FORTRAN program called NLMIX implementing the SNP method is available in the public domain. However, the experience with this approach is still very limited and only a few simulations have evaluated the ability of the method to reveal multiple modes or bumps in the random effects density under conditions likely to be encountered in practice [63]. The method has been used to analyze two data sets that were previously analyzed with the FO approach: phenobarbital pharmacokinetics in neonates [18,63] and quinidine pharmacokinetics in hospitalized male patients [60,65]. For phenobarbital, similar

parameter estimates were found when the SNP ap-

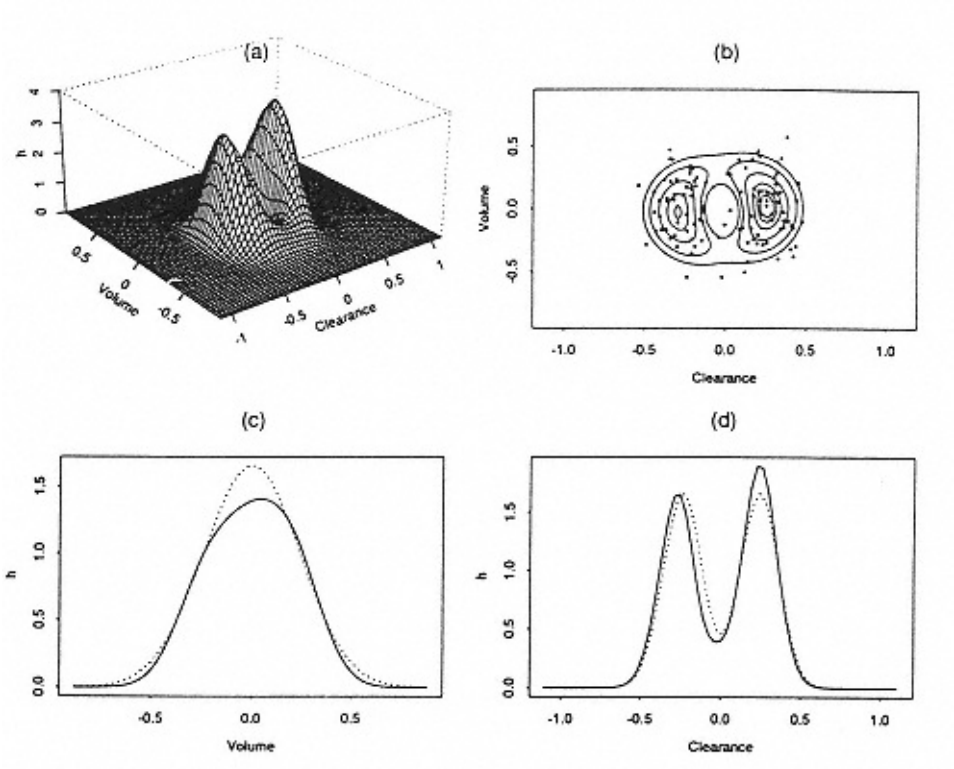


Figure 8

SNP estimate of the distribution of the interindividual random effect parameters (h) for the pharmacokinetic parameters clearance (Cl) and volume of distribution (V). Data were generated for 110 individuals according to a one-compartment pharmacokinetic model. The number of samples for each individual was randomly selected from 1 to 5. The interindividual variability in clearance and volume of distribution was specified by $\log(f_i) = \log(q) + h_i$, where $f_i = \{Cl_i, V_i\}^T$ and $q = \{0.1, 0.5\}^T$. Bi-modal random effects densities F(h) were generated by mixing two normal distributions $N(\mu, RRT)$ and $N(-\mu RRT)$ with mixing proportion 0.5 and $\mu = \{0.24, 0\}^T$. The elements of the upper triangular matrix R determine the coefficients of variation for the pharmacokinetic parameters. The elements of R were set to: $r_{11} = 0.12$, $r_{12} = 0$, and $r_{22} = 0.24$, yielding coefficients of variation between 25% and 28% for Cl and V. (a) The SNP estimate of the joint density of the interindividual random effect parameter h. (b) A contour plot of the SNP-estimated joint density at quantiles 10%, 25%, 50%, 75%, 90%, and 95% and the simulated random effects (dots). (c) and (d) The theoretical (dotted line) and SNP estimated (solid line) marginal distributions of the interindividual random effects parameters for V (left panel) and Cl (right panel). (From Reference 63.)

proach was constrained to a multivariate normal. However, the SNP method indicated a bimodality in the volume of distribution when the distribution assumptions were relaxed. For quinidine only a qualitative comparison can be made because of the different interindividual regression models that were used. Both methods indicated similar covariates that significantly influenced the clearance of quinidine.

Fattinger et al. [66] recently proposed a method similar to the SNP method to explore the complete distribution of interindividual effects using the FOCE approach in the NONMEM program. This method uses a monotone nondecreasing spline to transform the normally distributed interindividual random effects. The interindividual random effect model is expressed as

$$f_i = g(q, X_i) + sp(h_i) \quad (37)$$

where $sp(..)$ represents a monotone nondecreasing spline of which the parameters are estimated. A different spline is used for each of the elements of h_i because the spline is not multivariate. The spline transformation is very flexible and allows appropriate representations of skewed, heavy tail, or multimodal distributions. Fattinger et al. [66] compared the performance of their method with the SNP method and concluded that the SNP method has difficulties in estimating skewed densities, probably due to the use of polynomials in the representation for the density. The SNP method will produce apparent multimodality to account for skewness, whereas the spline transformation does not have this limitation.

4. Value of Mixed-Effects Modeling

The biggest contribution of mixed-effects modeling approaches to pharmacokinetic and pharmacodynamic data analysis has already been highlighted in the previous sections. These methods have made a completely new source of data accessible for analysis: so-called observational data. Observational data can be defined simply as data that have very few design restrictions [67] and are collected during routine clinical patient care or as a supplement in a study designed for another purpose. The characteristics of this type of data, namely small number of measurements, imbalance, and possibly confounding correlations, severely limit the application of pooled analysis and two-stage analysis techniques. However, mixed-effects modeling approaches have been shown to analyze observational data correctly.

The use of observational data has certain advantages that have been reviewed previously [20,67]. In general, the subjects contributing to the data are a better representation of the actual patient population treated with the drug. Thus, the derived population parameters are very relevant to optimizing treatment of the actual patients. Observational data are

relatively cheap because they are collected during routine clinical care or as a addition to a large clinical trial. Although

the data are sparse with respect to a certain individual, normally a large number of subjects are evaluated. This clearly favors the precision of the estimate of the distribution of the pharmacokinetic or pharmacodynamic parameters [30,68]. It will also increase the power to establish relationships between patient-specific covariates and the model parameters and discover unexpected factors that can influence drug disposition and action. The biggest disadvantage of observational data is that the quality of the data is not always controlled, especially if the data are collected retrospectively. If the study is performed in outpatients, compliance may be an important confounding factor. Antal et al. [10] clearly showed the impact of the quality of the data on the ability to determine population pharmacokinetic parameters for alprazolam using fragmentary data collected during clinical efficacy and safety trials. Analysis of prospectively collected data with accurate dosing and blood sampling time data yielded pharmacokinetic parameters identical to previously reported values. However, the analysis of retrospectively collected data that assumed strict adherence to protocol guidelines yielded seemingly precise but biased estimates of the mean parameters and markedly upward biased estimates of both residual and interindividual variability.

Nonlinear mixed-effects modeling is not restricted to observational data. In certain instances mixed-effects approaches have a number of important advantages for the analysis of experimental data. The first of these is obvious. It occurs when the experimental design calls for a sparse measurement scheme. This may be the case because of physical limitations to the number of measurements that can be obtained (e.g., pharmacokinetic studies in neonates or preclinical pharmacokinetic and toxicokinetic studies in small laboratory animals). Another reason may be the costs associated with extensive sampling. Several simulation studies have evaluated the impact of study design on the outcome of such sparse experimental data sets. The key to designing experiments with sparse sampling schemes is heterogeneity (e.g., varying the times of measurement among the individuals) [68].

Another advantage of mixed-effects approaches arises when estimates of the distribution of the interindividual random effects are of particular importance. As previously discussed, variability estimates are important for initial dosage selection and Bayesian dosage individualization. The interindividual variability may also be an important index of drug safety [14,67]. The smaller the unexplained variability, the more likely that a high percentage of individuals will receive a safe and efficacious treatment. The interindividual variability may be correlated to a certain covariate, for instance, with increasing age the mean response of the population may not be different, but the variability around the mean may be higher among older people. When the data arise from a well-controlled experiment with extensive sampling, the improved two-stage ap-

proaches such as the global two-stage or Bayesian two-stage may also be used to estimate the population parameters.

Yet another advantage of mixed effects approaches arises when one is interested in combining the results of several different studies. Mixed-effects modeling approaches allow different magnitudes of residual variability to be estimated for the data from different sources. This may be necessary to combine the studies appropriately, because the precision of the data obtained may differ from study to study. Especially in the early phases of drug development, one may want to combine the data of a series of small studies to estimate appropriately the population mean parameters and their interindividual variability required to design the following phases of drug development.

Mixed-effects modeling methods may be used in situations when the traditional analysis techniques become inappropriate due to loss of balance, confounding correlation (e.g., the number of samples in each individual correlates with the individual's half-life), or the fact that not every individual can be characterized with the same structural model (e.g., not every subject reaches E_{max} in a pharmacodynamic study).

Several interesting suggestions have been made on how specifically to design studies to make use of the advantages of mixed-effects modeling approaches and increase the efficiency of drug development [1015]. One of these suggestions is the dose-escalation design [69,70]. In a dose-escalation design the subjects receive increasing dosages of the drug until a clinical endpoint is reached. Once the endpoint is met the subjects are maintained on that dosage until the end of the study. This specific design has an obvious confounding correlation because only the least-sensitive subjects will receive the highest doses. If analyzed with a traditional (analysis of variance) method this will yield biased estimates of the pharmacodynamic parameters [69]. However, when analyzed with a mixed-effects approach unbiased estimates of the population parameters can be obtained with a precision comparable to that available from the gold-standard Latin square crossover design [69,70]. The dose-escalation design has some desirable features, namely, it resembles clinical practice and avoids ethical problems of over- and underdosing certain groups of patients. Figure 9 shows the population mean dose-response curve for the blood-pressure-lowering effect of atenolol derived using a dose-escalation study and the FO approach available in the NONMEM program. The figure shows that with increasing dosage the population mean starts to deviate from the mean of the data to compensate for the fact that the high-dosage data only consist of the least-sensitive patients.

The application of a full kinetic screen has been another suggestion to make use of the advantages of mixed-effects modeling approaches a drug development [15]. The full

screen suggests that one add a sparse sample design pharmacokinetic study to phase III efficacy and safety studies. The data can be used to derive population pharmacokinetic parameters in the target patient population.

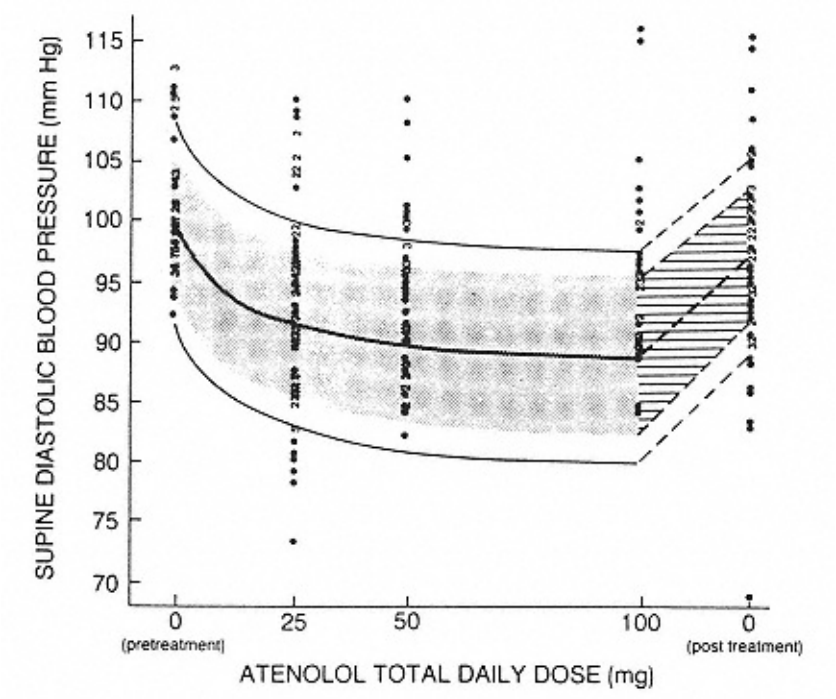


Figure 9
Dose versus supine diastolic blood pressure relationship for the b-adrenergic blocking agent atenolol. The data were derived from a multicenter dose-escalation study in 146 men and women who had histories of mild to moderate essential hypertension. The patients started on 25 mg atenolol by mouth once daily and were evaluated every two weeks. The dosage was doubled at the end of 4 weeks if the supine diastolic pressure was not controlled, up to a maximum of two doublings. The patients were maintained on the final dosage for an additional 12 weeks, followed by a 4 week placebo washout phase. An E^{max} model was fitted to the data (solid circles, or number if data are stacked). The bold center line represents the estimated curve for the mean individual using the FO approach in the NONMEM program. The shaded area is ±1 standard deviation of interindividual variability of response; the area bounded by the outer lines is ±1 standard deviation of interindividual plus residual variability of response (From Reference 69.)

In a more broad sense, the additional plasma samples would add significantly to the currently sparse information gathered on the relationship between pharmacodynamic response and plasma concentrations. The advantages and disadvantages of such an approach have been discussed [11,15] and the practical application has been evaluated [10,12].

Mixed-effect modeling methods may also be useful for interpreting dose-effect and concentration-effect studies according to a parametric pharmacokinetic-pharmacodynamic model [14]. Instead of focusing on the rejection of the null hypothesis that the drug does not work, one may want to try to understand the way in which drug dosage relates to effect and what factors influence this relationship [71]. A good example is the study on the population pharmacodynamic model for cognitive effect in patients with Alzheimer's disease treated with tacrine [46,72]. Standard analysis of variance showed a difference between the tacrine-treated patients and the placebo-treated group, but, due to limitations of these analysis techniques, only a subgroup of the studied population could be analyzed. Using the FO approach available in the NONMEM program, a population pharmacodynamic model was derived that described the disease progression without treatment, the placebo effect, and the effect of tacrine as a function of daily dosage. Furthermore, all available observations from one or more trials could be combined. The model confirmed the results of the traditional analysis of variance that tacrine has an effect that could be differentiated from placebo. However, the model can also be used to answer other questions such as whether the effect is proportional to dosage. The model also allows some explanation of the time course of disease and response to treatment and may be helpful, through simulations, to plan further clinical investigations. Similar examples can be found in other population studies [12,47].

IV. Conclusion

In this chapter the definitions and statistical theory of population pharmacokinetics and pharmacodynamics have been discussed. Several methods and software programs to obtain population parameter estimates have been reviewed and the role of these methods in clinical pharmacology and drug development has been briefly discussed. Population pharmacokinetics and pharmacodynamics is a quickly growing area, mostly because of the new methods that are being developed to estimate population parameters from sparse data sets. The potential of these new methods in clinical pharmacology and drug development is appealing. This has resulted in an increased interest for these approaches in the academic, regulatory, and industrial communities.

References

1. L. Aarons. Population pharmacokinetics: Theory and practice. Br. J. Clin. Pharmacol. 32:669670, 1991.

2. F. Mentre and A. Mallet. Experiences with NPML-application to dosage individualisation of cyclosporine, gentamicin and zidovudine. In New Strategies in Drug Development and Clinical Evaluation: The Population Approach. Edited by M.

Roland and L. Aarons. Commission of European Communities, Luxembourg, pp. 7590, 1992.

3. S. Vozeh and J. L. Steimer. Feedback control methods for drug dosage optimization. Concepts, classification and clinical application. Clin. Pharmacokinet. 10:457476, 1985.

4. M. Roland and L. Aarons. New Strategies in Drug Development and Clinical Evaluation: The Population Approach. Commission of European Communities, Luxembourg, 1992.

5. S. L. Beal and L. B. Sheiner. Estimating population kinetics. Crit. Rev. Biomed. Eng. 8:195222, 1982.

6. L. B. Sheiner, S. L. Beal, B. Rosenberg, and V. V. Marathe. Forecasting individual pharmacokinetics. Clin. Pharmacol. Ther. 26:294305, 1979.

7. L. B. Sheiner and S. L. Beal. Bayesian individualization of pharmacokinetics: simple implementation and comparison with non-Bayesian methods. J. Pharm. Sci. 71: 13441348, 1982.

8. S. Vozeh and C. Steiner. Estimates of the population pharmacokinetic parameters and performance of Bayesian feedback: a sensitivity analysis. J. Pharmacokinet. Biopharm. 15:511528, 1987.

9. B. Whiting, A. W. Kelman, and J. Grevel. Population pharmacokinetics. Theory and clinical application. Clin. Pharmacokinet. 11:387401, 1986.

10. E. J. Antal, T. H. Grasela, Jr., and R. B. Smith. An evaluation of population pharmacokinetics in therapeutic trials. Part III. Prospective data collection versus retrospective data assembly. Clin. Pharmacol. Ther. 46:552559, 1989.

11. W. A. Colburn, T. H. Grasela, E. J. Antal, S. C. Olson, C. T. Viswanathan, and J. P. Skelly. Controversy IV: Population pharmacokinetics, NONMEM and the pharmacokinetic screen; academic, industrial and regulatory perspectives. J. Clin. Pharmacol. 29:16, 1989.

12. T. H. Grasela, Jr., E. J. Antal, L. Ereshefsky, B. G. Wells, R. L. Evans, and R. B. Smith. An evaluation of population pharmacokinetics in therapeutic trials. Part II. Detection of a drug-drug interaction. Clin. Pharmacol. Ther. 42:433441, 1987.

13. T. H. Grasela, Jr., E. J. Antal, R. J. Townsend, and R. B. Smith. An evaluation of population pharmacokinetics in therapeutic trials. Part I. Comparison of methodologies. Clin. Pharmacol. Ther. 39:605612, 1986.

14. L. B. Sheiner. Population approach in drug development: Rationale and basic concepts. In New Strategies in Drug Development and Clinical Evaluation: The Population Approach. Edited by M. Roland and L. Aarons. Commission of European Communities, Luxembourg, pp. 1329, 1992.

15. L. B. Sheiner and L. Z. Benet. Premarketing observational studies of population pharmacokinetics of new drugs. Clin. Pharmacol. Ther. 38:481487, 1985.

16. L. Collart, T. F. Blaschke, F. Boucher, and C. G. Prober. Potential of population pharmacokinetics to reduce the frequency of blood sampling required for estimating kinetic parameters in neonates. Dev. Pharmacol. Ther. 18:7180, 1992.

17. M. S. Driscoll, T. M. Ludden, D. T. Castro, and L. C. Littlefield. Evaluation of theophylline pharmacokinetics in a pediatric population using mixed effects models. J. Pharmacokinet. Biopharm. 17:141168, 1989.

18. T. H. Grasela, Jr. and S. M. Donn. Neonatal population pharmacokinetics of phenobarbital derived from routine clinical data. Dev. Pharmacol. Ther. 8:374383, 1985.

19. L. B. Sheiner. Analysis of pharmacokinetic data using parametric models. II. Point estimates of an individual's parameters. J. Pharmacokinet. Biopharm. 13:515540, 1985.

20. L. B. Sheiner and T. M. Ludden. Population pharmacokinetics/dynamics. Annu. Rev. Pharmacol. Toxicol. 32:185209, 1992.

21. L. B. Sheiner. Analysis of pharmacokinetic data using parametric models. I. Regression models. J. Pharmacokinet. Biopharm. 12:93117, 1984.

22. J. B. Dyck, M. Maze, C. Haack, D. L. Azarnoff, L. Vuorilehto, and S. L. Shafer. Computer-controlled infusion of intravenous dexmedetomidine hydrochloride in adult human volunteers. Anesthesiology 78:821828, 1993.

23. T. D. Egan, H. J. Lemmens, P. Fiset, D. J. Hermann, K. T. Muir, D. R. Stanski and S. L. Shafer. The pharmacokinetics of the new short-acting opioid remifentanil (GI87084B) in healthy adult male volunteers. Anesthesiology 79:881892, 1993.

24. L. L. Gustafsson, W. F. Ebling, E. Osaki, S. Harapat, D. R. Stanski, and S. L. Shafer. Plasma concentration clamping in the rat using a computer-controlled infusion pump. Pharm. Res. 9:800807, 1992.

25. B. K. Kataria, S. A. Ved, H. F. Nicodemus, G. R. Hoy, D. Lea, M. Y. Dubois, J. W. Mandema, and S. L. Shafer. The pharmacokinetics of propofol in children using three different data analysis approaches. Anesthesiology, 80:104122, 1994.

26. S. L. Shafer, J. R. Varvel, N. Aziz, and J. C. Scott. Pharmacokinetics of fentanyl administered by computer-controlled infusion pump. Anesthesiology 73:10911102, 1990.

27. C. C. Peck, D. Z. D'Argenio, and J. H. Rodman. Analysis of pharmacokinetic data for individualizing drug dosage regimens. In Applied Pharmacokinetics. Principles of Therapeutic Drug Monitoring. Edited by W. E. Evans, J. J. Schentag, and W. J. Jusko. Applied Therapeutics, Vancouver, 1992.

28. L. B. Sheiner and S. L. Beal. Evaluation of methods for estimating population pharmacokinetics parameters. II. Bioexponential model and experimental pharmacokinetic data. J. Pharmacokinet. Biopharm. 9:635651, 1981.

29. J. L. Steimer, A. Mallet, J. L. Golmard, and J. F. Boisvieux. Alternative approaches to estimation of population pharmacokinetic parameters: comparison with the nonlinear mixed-effect model. Drug Metab. Rev. 15:265292, 1984.

30. L. B. Sheiner and S. L. Beal. Evaluation of methods for estimating population pharmacokinetic parameters. III. Monoexponential model: routine clinical pharmacokinetic data. J. Pharmacokinet. Biopharm. 11:303319, 1983.

31. S. L. Beal. Population pharmacokinetic data and parameter estimation based on their first two moments. Drug Metabol. Rev. 15:173193, 1984.

32. A. Racine-Poon. A Bayesian approach to nonlinear random effects models. Biometrics 41:10151023, 1985.

33. L. B. Sheiner. Analysis of pharmacokinetic data using parametric models. III. Hypothesis tests and confidence intervals. J. Pharmacokinet. Biopharm. 14:539555, 1986.

34. L. B. Sheiner and S. L. Beal. A note on confidence intervals with extended least squares parameter estimates. J. Pharmacokinet. Biopharm. 15:9398, 1987.

35. D. B. White, C. A. Walawander, Y. Tung, and T. H. Grasela. An evaluation of point and interval estimates in population pharmacokinetics using NONMEM analysis. J. Pharmacokinet. Biopharm. 19:87112, 1991.

36. A. P. Dempster, N. M. Laird, and D. B. Rubin. Maximum likelihood from incomplete data via the EM algorithm. J. Roy. Stat. Soc. B, 39:138, 1977.

37. M. Pitsiu, E. M. Parker, L. Aarons, and M. Rowland. Population pharmacokinetics and pharmacodynamics of warfarin in healthy young adults. Eur. J. Pharm. Sci. 1:151157, 1993.

38. A. Racine, A. P. Grieve, H. Fluhler, and A. F. M. Smith. Bayesian methods in practice: Experiences in the pharmaceutical industry. Appl. Stat. 35:93150, 1986.

39. A. Racine-Poon and J. Moppert. Oxprenolol: Concentration effect relationship. A retrospective study. Br. J. Clin. Pharmacol. 19:143S149S, 1985.

40. A. Racine-Poon and A. F. M. Smith. Population models. In Statistical Methodology in the Pharmaceutical Science. Edited by D. Berry. New York, Marcel Dekker, 1990, pp. 139162.

41. M. J. Lindstrom and D. M. Bates. Nonlinear mixed effects models for repeated measures data. Biometrics, 46:673687, 1990.

42. E. F. Vonesh and R. L. Carter. Mixed-effects nonlinear regression for unbalanced repeated measures. Biometrics 48:117, 1992.

43. L. B. Sheiner and S. L. Beal. Evaluation of methods for estimating population pharmacokinetics parameters. I. Michaelis-Menten model: Routine clinical pharmacokinetic data. J. Pharmacokinet. Biopharm. 8:553571, 1980.

44. L. Aarons, J. W. Mandema, and M. Danhof. A population analysis of the pharmacokinetics and pharmacodynamics of midazolam in the rat. J. Pharmacokinet. Biopharm. 19:485496, 1991.

45. D. A. Graves, K. T. Muir, W. Richards, B. W. Steiger, I. Chang and B. Patel. Hydralazine dose-response curve analysis. J. Pharmacokinet. Biopharm. 18:279291, 1990.

46. N. H. Holford and K. E. Peace. Results and validation of a population pharmacodynamic model for cognitive effects in Alzheimer patients treated with tacrine. Proc. Natl. Acad. Sci. U.S.A. 89:1147111475, 1992.

47. N. H. Holford, Y. Hashimoto, and L. B. Sheiner. Time and theophylline concentration help explain the recovery of peak flow following acute airways obstruction. Clin. Pharmacokinet. 25:506515, 1993.

48. N. C. Sambol and L. B. Sheiner. Population dose versus response of betaxolol and atenolol: A comparison of potency and variability. Clin. Pharmacol. Ther. 49:2431, 1991.

49. D. R. Stanski and P. O. Maitre. Population pharmacokinetics and pharmacodynamics of thiopental: The effect of age revisited. Anesthesiology 72:412422, 1990.

50. N. Kaniwa, N. Aoyagi, H. Ogata, and M. Ishi. Application of the NONMEM method to evaluation of the bioavailability of drug products. J. Pharm. Sci. 79: 11161120, 1990.

51. S. M. Pai, U. A. Shukla, T. H. Grasela, C. A. Knupp, R. Dolin, F. T. Valentine, C. McLaren, H. A. Liebman, R. R. Martin, and K. A. Pittman. Population pharma-

cokinetic analysis of didanosine (2',3'-dideoxyinosine) plasma concentrations obtained in phase I clinical trials in patients with AIDS or AIDS-related complex. J. Clin. Pharmacol. 32:242247, 1992.

52. S. L. Beal and L. B. Sheiner. NONMEM Users GuidePart VII. Conditional estimation methods. University of California, San Francisco, 1992.

53. N. G. De Bruijn. Asymptotic Methods in Analysis. Amsterdam, The Netherlands, 1961.

54. P. O. Maitre, M. Buhrer, D. Thomson, and D. R. Stanski. A three-step approach combining Bayesian regression and NONMEM population analysis: application to midazolam. J. Pharmacokinet. Biopharm. 19:377384, 1991.

55. J. W. Mandema, D. Verotta, and L. B. Sheiner. Building population pharmacokinetic-pharmacodynamic models. I. Models for covariate effects. J. Pharmacokinet. Biopharm. 20:511528, 1992.

56. C. C. Peck and B. C. Chen. Importance of assumptions in Bayesian pharmacokinetic control of drug therapy. Clin. Pharmacol. Ther. 37:220, 1985.

57. A. Mallet, F. Mentre, J. Gilles, A. W. Kelman, A. N. Thomson, S. M. Bryson, and B. Whiting. Handling covariates in population pharmacokinetics with an application to gentamicin. Biomed. Meas. Inform. Contr. 2:673683, 1988.

58. A. Mallet. A maximum likelihood estimation method for random coefficient regression models. Biometrika 73:645656, 1986.

59. J. L. Steimer, A. Mallet, and F. Mentre. Estimating inter-individual pharmacokinetic variability. In Variability in Drug Therapy: Description, Estimation, and Control. Edited by M. Rowland et al., Raven Press, New York, 1985, pp. 65111.

60. M. Davidian and A. R. Gallant. Smooth nonparametric maximum likelihood estimation for population pharmacokinetics, with application to quinidine. J. Pharmacokinet. Biopharm. 20:529556, 1992.

61. A. Mallet, F. Mentre, J. L. Steimer, and F. Lokiec. Nonparametric maximum likelihood estimation for population pharmacokinetics, with application to cyclosporine. J. Pharmacokinet. Biopharm. 16:311327, 1988.

62. F. Mentre, A. Mallet, B. Diquet, P. Turk, J. N. Collin, and P. Dowd. Population kinetics of AZT in AIDS patients. Eur. J. Clin. Pharmacol. 36:230, 1989.

63. M. Davidian and A. R. Gallant. The nonlinear mixed effects model with a smooth

random effects density, Institute of Statistics Mimeo Series No. 2206 North Carolina State University, Raleigh, NC, 1992.

64. A. R. Gallant and D. W. Nychka. Seminonparametric maximum likelihood estimation. Econometrica 55:363390, 1987.

65. C. N. Verme, T. M. Ludden, W. A. Clementini, and S. C. Harris. Pharmacokinetics of quinidine in male patients. A population analysis. Clin. Pharmacokinet. 22: 468480, 1992.

66. K. E. Fattinger, L. B. Sheiner, and D. Verotta. A new method to explore the distribution of interindividual random effects in nonlinear mixed effects models. Technical report #33, Department of Epidemiology & Biostatistics, University of California, San Francisco, 1993.

67. L. B. Sheiner and T. H. Grasela. An introduction to mixed effect modeling: Concepts, definitions, and justification. J. Pharmacokinet. Biopharm. 19:11S24S, 1991.

68. Y. Hashimoto and L. B. Sheiner. Designs for population pharmacodynamics: value of pharmacokinetic data and population analysis. J. Pharmacokinet. Biopharm. 19: 333353, 1991.

69. L. B. Sheiner, S. L. Beal, and N. C. Sambol. Study designs for dose-ranging. Clin. Pharmacol. Ther. 46:6377, 1989.

70. L. B. Sheiner, Y. Hashimoto, and S. L. Beal. A simulation study comparing designs for dose ranging. Stat. Med. 10:303321, 1991.

71. L. B. Sheiner. The intellectual health of clinical drug evaluation. Clin. Pharmacol. Ther. 50:49, 1991.

72. N. H. Holford and K. E. Peace. Methodologic aspects of a population pharmacodynamic model for cognitive effects in Alzheimer patients treated with tacrine. Proc. Natl. Acad. Sci. U.S.A. 89:1146611470, 1992.

15
Bioavailability and Bioequivalence of Oral Controlled-Release Products: A Regulatory Perspective

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I. Introduction.

In the previous edition of this book [1], Dighe and Adams described the requirements established by the Food and Drug Administration (FDA) to ensure the bioavailability and bioequivalence of extended-release products and requirements for dissolution. This updated chapter focuses on four major aspects of FDA's current review process for oral extended-release drug products: new concepts advanced by pharmaceutical associations and regulatory agencies from workshop reports; current biostudy and dissolution requirements for oral extended-release products and their generic versions; issues concerning the design and criteria for evaluation of biostudies; current and proposed requirements for postapproval modifications.

In accord with U.S. Pharmacopeia (USP) nomenclature [2], the term extended-release dosage forms will be used throughout this chapter.

II. Developments in Scientific and Regulatory Aspects

Since this chapter was originally published [1], there have been several workshop reports, USP proposals, and an Agency guidance with implications for or directly affecting the regulatory review process for extended-release products.

The USP [2] proposed an official definition of in vivo/in vitro correlation (IVIVC) for

extended-release dosage forms. Correlation procedures were categorized into levels A, B, C, and D [2]. A level A correlation demonstrates a 1:1 relationship between in vitro and in vivo dissolution such that their curves are superimposable. The in vivo dissolution curve may be obtained by model-dependent (Wagner-Nelson or Loo-Riegelman procedures) or -independent (mathematical deconvolution) methods. The level B correlation compares the

mean in vitro dissolution time to either the mean residence time or the mean in vivo dissolution time. The level C correlation relates one dissolution time to one pharmacokinetic parameter (single point correlation). The USP Subcommittee on Biopharmaceutics supported the level A correlation as a means of justifying postapproval product modifications (changes in manufacturing site, methods, sources of raw materials, and minor formulation changes) without additional human studies.

A report from the second workshop on controlled/modified release dosage forms [3] was issued regarding In Vitro and In Vivo Testing and Correlation for Oral Controlled/Modified-Release Dosage Forms. Recommendations were made concerning which pharmacokinetic studies were needed in several cases [3].

Case I is an extended-release oral dosage form of a marketed immediate-release drug for which extensive pharmacodynamic-pharmacokinetic (PD-PK) data exist. The two studies needed are:

1. Study 1: single-dose crossover with three treatments

a. Extended-release dosage form (fasting)

b. Rapidly available dosage form (fasting)

c. Extended-release dosage form (high-fat meal)

2. Study 2: multiple-dose, steady-state with two treatments

a. Extended-release dosage form

b. Immediate-release formulation

Depending on the results of the first study, additional studies may be required to define the cause(s) of food effects and the effects of time on the food-drug interaction. For study 1, no specific criteria were stated. For study 2, the area under the curve (AUC) for the extended-release product over one dosing interval should be equivalent to the AUC of the immediate-release product, and the degree of fluctuation should be less then or equal to that of the immediate-release product. Subject selection should be randomized or from the target population or specific subpopulation. Steady-state studies in patient populations and drug interaction studies may also be necessary.

Case II refers to nonoral extended-release dosage forms of drugs meeting the criteria in case I.

Case III is for generic equivalents of approved extended-release products. The generic formulation must be comparable with respect to rate and extent of availability (using the

parameters AUC, CMAX (peak concentration), CMIN (trough concentration), and fluctuation) to the standard extended-release product in a steady-state crossover study. The food studies as described in case I are also needed.

Case IV is for an extended-release dosage form as a new drug application (NDA). The dosage form should be characterized with regard to dose linearity, food effects, absorption (extent, rate, pattern), and fluctuation.

The workshop report also agreed in principle with the USP article on IVIVC [2] but recognized that separate correlations may be required for each manufacturer's extended-release product. The in vitro dissolution method and specifications must be optimized for response to the range of critical manufacturing variables that affect drug release. Methods for development and validation for IVIVC were described. These processes will be discussed below in the section on postapproval changes.

The USP proposed a chapter 1088 [4] In Vitro and In Vivo Evaluation of Dosage Forms based on a composite of the December, 1988 AAPS/FDA workshop report [3] and the Stimuli article on IVIVC [1]. The chapter describes the methods for in vitro and in vivo evaluation of extended-release dosage forms, and the methods for development of IVIVC and establishment of dissolution specification ranges. These issues were further discussed at the USP/HPB/FDA International Open Conference on Dissolution, Bioavailability, and Bioequivalence [5].

In September, 1992, the AAPS/FDA/USP held a workshop Scale-Up of Oral Extended Release Dosage Forms [6]. In a previous workshop [7] on scale-up of immediate-release dosage forms, certain formulation changes after scale-up were identified as acceptable without further consideration. The Workshop II (6) report stated that these same formulation changes appear to be equally applicable to extended-release products for noncritical components not affecting drug release. Changes in noncritical components within the ranges recommended for immediate-release dosage forms are deemed minor changes. Changes in non-critical components beyond the ranges recommended for the immediate-release products are deemed major changes and should be based on in vitro data if an IVIVC has been established. The total additive effect of excipients should not change the drug/excipient ratio by more than 5%. Changes in critical components affecting drug release are deemed major changes and must be justified based on IVIVC.

Alterations in the drug substance are considered major changes if aqueous solubility ≤ 5 mg/mL and a $> 10\%$ change in mean particle size, surface area, or dissolution rate; aqueous solubility > 5 mg/mL and a $> 25\%$ change in mean particle size, surface area, or dissolution rate. Processing and equipment changes after scale-up may be allowed if an IVIVC is established and the dissolution profile falls within the specifications set prior to scale-up, with no change in the release mechanism or qualitative composition. Minor process and equipment changes should be permitted based on a reliable, predictive dissolution test.

Dissolution, under the proper conditions, may serve as a surrogate for bioequivalence studies during scale-up or site changes. There was general agreement, with minor modifications, with the approach to IVIVC given in the proposed USP chapter 1088 [4].

Level A correlations can be used to establish dissolution data as a surrogate for bioequivalence data during batch size scale-

up of extended-release products. If dissolution rate is independent of testing conditions, the correlation may be used directly as a surrogate for additional in vivo studies. If dissolution rate is dependent on testing conditions, further validation is required. If a level A correlation cannot be obtained, the procedure of mapping may be used to validate dissolution specifications as a substitute for in vivo data.

The Division of Bioequivalence (DBE) in the Office of Generic Drugs (OGD), FDA, issued a guidance [8], Oral Extended (Controlled) Release Dosage Forms: In Vivo Bioequivalence and In Vitro Dissolution Testing. The biostudies and dissolution testing required to support an Abbreviated New Drug Application (ANDA) for an extended-release product are discussed in detail in Section IV. In addition to the biostudies and dissolution data, the following points are significant:

1. Although the generic product and the listed reference product must be pharmaceutically equivalent, the mechanism by which the release of active substance occurs does not have to be the same.

2. The biobatch used for in vivo bioequivalence studies must conform to the OGD Policy and Procedure Guide 2290 [9].

3. The assayed potencies of the test and reference products should not differ by more than 5%.

4. A single dose, two-way crossover fasting study is required for each strength of a generic extended-release tablet product. The multiple dose, steady-state study and the food study are conducted only on the highest strength.

5. A single dose, two-way crossover fasting study is required for only the highest strength of a generic extended-release capsule product, provided that:

a. Formula compositions of the lower strengths are proportional to that of this highest strength

b. The capsules contain identical beads or pellets.

Single-dose studies for the lower strengths may then be waived on the basis of acceptable dissolution testing. The multiple dose and food studies are to be conducted with the highest strength of the capsule formulation.

III. Biopharmaceutics Review of Novel Oral Extended-Release Products

The types of studies appropriate for the first extended-release (ER) product to be marketed for a particular drug will vary depending on several circumstances. The most

general classification involves classifying the ER products depending on whether or not the drug contained in the ER product has already been approved for marketing in a immediate-release (IR) form. In circumstances where

an IR product has been approved and is marketed, the safety and efficacy of the drug itself has been established and is not an issue. If however, there is no approved IR product for the new ER drug product, the complete array of clinical trials, appropriate for a new drug, with undetermined safety and efficacy, will need to be performed. If an approved IR product is available, the safety and efficacy of the ER *drug product,* containing a drug of established safety and efficacy in a new formulation, needs to be studied comprehensively.

Another important aspect of ER products in circumstances where no ER product has been approved but IR products are approved, is the need for clinical studies for the first ER product. While generic ER products would not be subjected to clinical safety and efficacy studies, since bioequivalency to the reference ER product is the primary goal, the first ER product frequently must be tested clinically to establish the safety and efficacy of this new and unique ER dosage form. Plasma concentration profiles for an ER product often will differ substantially from IR product profiles and this can raise questions related to clinical safety and efficacy.

These areas merit further discussion related to new ER drug product approval requirements.

A. ER Product Containing a Drug Approved and Marketed in IR Dosage Forms Only

1. In Vivo Bioavailability Studies

From a biopharmaceutics perspective, the primary objective is to define the ER dosage form's pharmacokinetic characteristics relative to an approved IR reference product administered according to its approved dosing [10]. The ER product usually contains multiples of the single IR dose, usually, two to four times the single IR dose. At the present time, the maximum possible oral dosage interval for an ER product is 24 h, due to limits in gastrointestinal transit, which can range from 5 to 36 h [11]. While the exact number of studies and study design may vary depending on factors such as the drug's pharmacokinetics, the studies generally will involve both single and multiple dose drug adminstration.

2. Single-Dose Crossover Studies

The purpose of single-dose studies for new ER dosage forms includes the definition of the effect of food on the release characteristics of the dosage form and the establishment of bioequivalence of each strength of the ER product, usually under fasting conditions.

The design and analysis of the bioequivalence study are the same as described for generic ER products in Section IV.

The study to determine food effects is designed to stress the dosage form to show the maximum possible effect, if there will, in fact, be an effect. For this

reason the usually recommended meal is not a typical meal but one with high fat content and high calorie content. For NDA submissions to the FDA, the recommended meal is high-fat and high-calorie and consists of two slices toasted bread with butter, two eggs fried in butter, two slices of bacon, 2 oz. hash brown potatoes, and 240 mL whole milk. This meal contains approximately 33 g protein (132 cal), 5875 g fat (522675 cal), 58 g carbohydrate (232 cal): total 8701020 calories.

This meal, sometimes referred to as the FDA high-fat breakfast, was not developed by the FDA but was the result of extensive testing by competing pharmaceutical manufacturers in the 1980s. In many of the studies, conducted on theophylline once-daily ER products, this meal was shown to be very effective in causing significant changes in bioavailability characteristics, both increase and decrease. While appropriate arguments have been made that this meal is not at all a typical breakfast, the intent is not as a typical meal but as a stresstest of the formulation. If a food study conducted using the FDA high-fat breakfast shows no significant effect, it is thought likely that no other meal is likely to elicit larger bioavailability changes.

This study has little or no bioequivalence aspects. Its primary purpose is to define the effect of food, if any. While it certainly is acceptable if the ER product, administered fasted and fed, is shown to be bioequivalent, this is not a requirement.

Meals can have an important impact on the bioavailability characteristics of drugs from both IR and ER dosage forms. This effect is complex and involves factors such as gastrointestinal transit, dosage form stability, and meal content. An excellent review of this area has been prepared [12].

For IR products, one of the most significant effects, in this regard, is for atovaquone. From the labeling, the degree of the effect and the importance of administering the drug with food are made very clear. In the pharmacokinetics section of the labeling the food study results are described:

> The bioavailability of atovaquone is increased approximately 3-fold when administered with meals. In particular, fat has been shown to enhance absorption significantly. In one study, 18 volunteers received a single dose of 500 mg atovaquone after an overnight fast and following breakfast (23 g fat: 642 kcal). The mean (+/- SD) AUC values were 93.8 +/- 45.7 and 288 g +/- 77 hr µg/mL, under fasting and fed conditions, respectively. In another volunteer study where atovaquone was administered under fasting conditions, with 28g butter (23g fat) and 56g butter (46g fat) on toast, mean AUC values increased 2.7- and 4.0- fold, respectively, compared to the fasting state.

The importance of taking atovaquone with food is described in the dosage and administrative section: Failure to administer atovaquone with food may result in lower

atovaquone plasma concentrations and may limit response to therapy.

For ER products, at issue is the potential rapid release of drug at a much faster rate than expected. This is often referred to as dose-dumping. The immediate release of all drug in an ER dosage form due to formulation failure could raise serious safety concerns. The high-fat meal has also been shown to decrease bioavailability from some ER products [12].

In most circumstances the administration of an ER dosage form with food does cause some small to moderate change in the bioavailability characteristics. For new ER products, the results of the food-effect study are described in the product's labeling. The study is usually not subject to pass-fail statistical criteria and the product only would be thought to fail if very large effects (increase or decrease) are shown, rendering the product potentially unsafe or ineffective. Some examples will serve to illustrate typically how study results are described in product labeling.

The results of administering a diltiazem ER product with a high-fat breakfast are shown in Figure 1.

The results of this study are summarized in the pharmacokinetics section of the labeling:

> The presence of food did not affect the ability of [diltiazem ER] to maintain a continuous release of drug for up to 24 hours after administration. Simultaneous administration with a high-fat breakfast had a modest effect on diltiazem bioavailability with AUC increasing by 13% and Cmax by 37%.

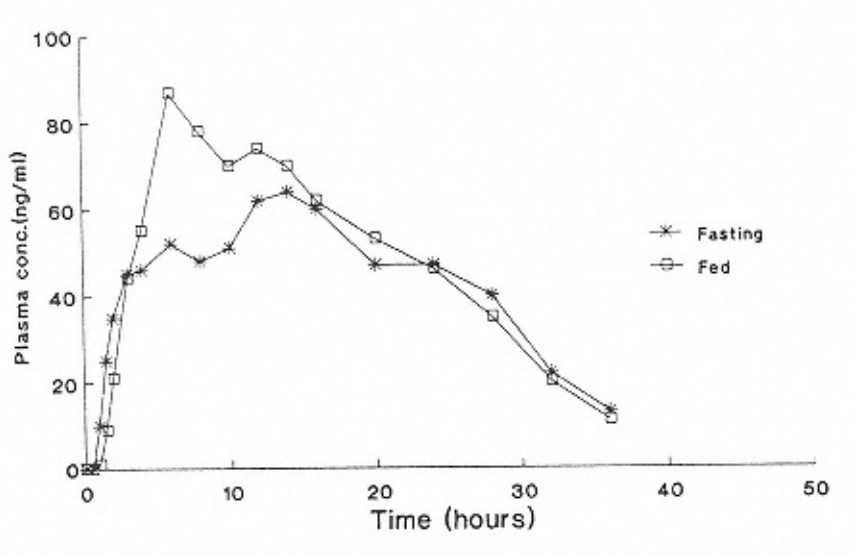

Figure 1
Diltiazem ER product under fed and fasting conditions.

The dosage and administration section recommends that the product be administered without food: Studies have shown a slight increase in the rate of absorption, when ingested with a high-fat breakfast; therefore, administration in the morning on an empty stomach is recommended.

A nicardipine ER product was tested at steady state using a high-fat meal. The results are summarized in Figure 2.

The study results are summarized in the pharmacokinetics section of the labeling:

> When [nicardipine ER] was administered with a high-fat breakfast, mean Cmax was 45% lower, AUC was 25% lower, and trough levels were 75% higher than when [nifedipine ER] was given in the fasting state. Thus taking [nifedipine ER] with the meal reduced the fluctuation in plasma levels. Clinical trials establishing the safety and efficacy of [nicardipine ER] were carried out in patients without regard to the timing of meals.

No restrictions with regard to administration of this product with meals are mentioned in the Dosage and Administration section of the labeling. These examples illustrate that bioequivalence is not a requirement for food-effect studies in these circumstances and also show how the study results may be reflected in product labeling. If significant effects of food are shown, it has been suggested [13] that additional studies might be warranted, to define the cause of the effect and also the effect of meal/drug timing on the effect. An important recommen-

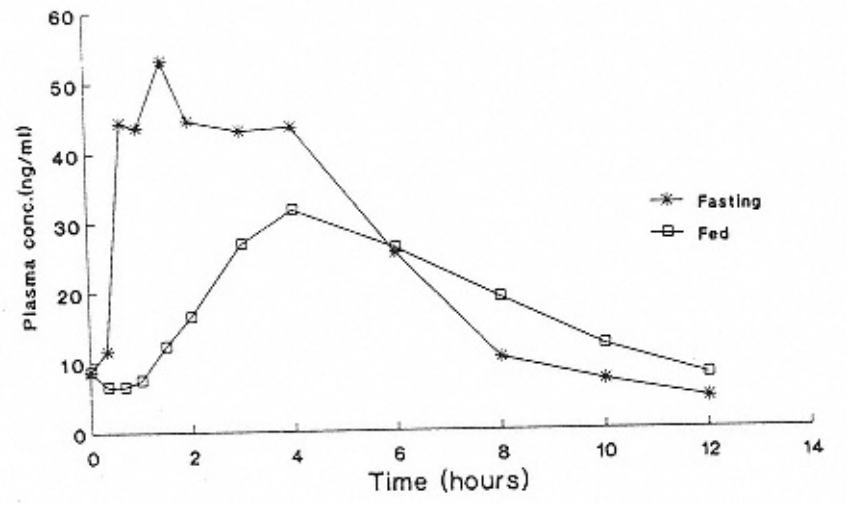

Figure 2
Nicardipine ER product under fed and fasting conditions.

dation is that the effect of food be determined (this recommendation can also be made for IR products). If significant effects are shown, consideration should be given to controlling meals in relation to drug administration during clinical trials establishing safety and efficacy, in order to optimize dosage form release characteristics and bioavailability. It is thought that such early information regarding the effect of food on bioavailability can be used to minimize variability in bioavailability and perhaps contribute significantly to the determination of safety and efficacy where food significantly increases or decreases bioavailability.

3. Multiple Dose Studies

The purpose of a multiple-dose study for a new ER drug product is to determine the relative concentration-time profiles for the new ER product compared to an established IR product administered according to its labeling [10]. Bioequivalence of the ER product to the IR product is not an essential requirement and, depending on one's definition of bioequivalence, may not be, in fact, attainable regarding the same rate of absorption. ER products are usually designed to alter the rate of absorption compared to an IR product intended for rapid absorption. Establishment of bioequivalence of an ER product to an approved IR product, or, on the contrary, results indicating lack of such equivalence, can have a direct bearing on the need for additional clinical safety and efficacy data for approval of the ER product.

The design and analysis of the multiple-dose study are the same as described in Section IV for generic ER products, except that the reference product is an approved IR product.

The Workshop Report [13] suggests the need to conduct the multiple dose study at two different dose rates, one at the low end of the recommended dosing range, and second at the high end, if the drug involved is known to have nonlinear kinetics (or if such data are not available). This reflects current FDA policy for the approval of new ER products.

4. Additional Studies

The need for additional studies depends primarily on how well the drug contained in the ER dosage form has been characterized. These studies may involve, for example, further study of the drug's metabolism from the ER dosage form, effect of day/night dosing or further definition of a food-effect, if a significant effect is seen in the study described above.

B. ER Product Containing a Drug Not Previously Approved as Safe and Effective

Occasionally an NDA for an ER dosage form is submitted for a drug that is unapproved. That is, no immediate release dosage form has been approved and

marketed. In these circumstances all pharmacokinetic and bioavailability studies appropriate for a new chemical entity are necessary, in addition to the studies described in Section III. A. These include determination of the essential pharmacokinetics, dose proportionality, and special population studies (for example, elderly, gender, impaired renal and hepatic function). A comparison to a reference formulation for purposes of bioequivalence is not needed, but determination of bioavailability relative to an oral or intravenous solution is appropriate. Table 1 illustrates the types of studies appropriate for such a product.

C. Additional Considerations

1. Study Involving Change in Drug Release Rate.

Another important study, often seen as part of an NDA submission for a new ER drug product, is a single-dose three-way crossover study, which involves administration of three variations of the ER product's formulation. These formulations differ in drug release rate and are the result of variation in the release-controlling excipients of the formulation. This study may involve a relatively small number of subjects (for example, six to nine) and is usually not subject to strict statistical analysis.

Table 1 Types of Studies: ER Product with no Approved IR Product

Biotransformation
Protein binding
Bioavailability (oral solution)
Absolute bioavailability
Site of absorption (gut wall metabolism)
Assessment of stereoisomer pharmacokinetics
Dose proportionality
Steady-state pharmacokinetics
Bioequivalence of different dosage strengths
Effect of formulation change on bioavailability
Comparison of formulations with different release rates
Influence of food on bioavailability
Effect of age
Effect of gender
Effect of impaired hepatic function
Effect of impaired renal function
Drug interaction studies
Pharmacokinetics in patients (single-dose and multiple-dose)
In vitro/in vivo correlation

Dissolution testing

This study is an important part of the development of the final formulation for the product. The formulation that shows optimal controlled-release characteristics, when the results of this study are evaluated, then goes forward in the pivotal studies described earlier, as well as in clinical safety and efficacy studies.

Another important use of this study is in the development and validation of an in vitro/in vivo correlation for the product.

2. In Vitro/In Vivo Correlation

A correlation should be an essential consideration in the development of all ER drug products. This issue has been discussed for many years and was well-presented in both ER Workshops Reports [13,14] as well as a USP Pharmacopeial Forum article [2] and a recent USP chapter [15].

Dissolution testing is widely used as a quality control test for both IR and ER drug products. More than 500 dissolution tests appear in monographs in USP 23. However, relatively few in vitro/in vivo correlations have been developed. Such correlations directly relate in vitro dissolution rate to in vivo dissolution or absorption rate. This can allow the prediction of bioavailability characteristics for batches of drug product that have not undergone in vivo testing.

It is likely that correlations, in general, are more feasible for ER products, since for ER products, in vivo dissolution (following release from the dosage form) is the key determinant for drug absorption and the prolonged plasma concentrations seen with ER products. In order for correlations to be more likely to be seen, modification in current dissolution testing procedures may be necessary. These modifications would relate to attempting to make the in vitro dissolution test conditions more physiological particularly as related to pH, surface tension, agitation speed, and volume of medium. It would seem reasonable that dissolution test conditions involving changing pH during the test, from acid to neutral, would greatly improve the relationship of in vitro dissolution conditions to in vivo physiological conditions for ER products and perhaps IR products also.

In an attempt to determine the extent of correlations submitted in NDAs, a survey [16] of original NDAs for ER products approved by the FDA from 1983 to 1993 revealed that 57 NDAs were approved and these submissions contained 9 attempts at correlations. Nearly all of these correlations were for cardiac drugs, indicating mostly that numerous ER products for cardiac drugs were approved in the time period.

Correlation attempts as part of ANDAs for generic drugs have been rare, with only one recent ANDA submission being known to have included a correlation.

The general scarcity of correlations found in the FDA survey is nearly completely the result of no correlation being attempted (or submitted) rather than a failed attempt at correlation. Most of the correlations found in the survey were

used in the process of setting the dissolution specifications for the products. In one instance the correlation was used to allow waiver of bioequivalency testing for a postapproval formulation change. Without this correlation in this instance, an in vivo bioequivalence study would have been needed.

A question frequently asked is Is there an in vitro/in vivo correlation? A yes or no answer is anticipated but further thought reveals that correlations involve three areas: development, validation, and application. Only by thinking in this regard do all aspects of correlations become apparent.

Development of a correlation involves testing the predictive abilities of the correlation and therefore is an important and essential aspect. An unvalidated correlation is of little value as a reliable surrogate for bioequivalency. Correlation validation normally involves the conduct of at least one other bioavailability/bioequivalency study, using data not involved in correlation development, but other potential methods might be applicable, such as cross-validation and bootstrapping [17,18]. These computer-intensive statistical procedures are being studied as a possible means of further reducing the amount of in vivo data required for a validated correlation.

In regard to the application or use of correlations, the setting of dissolution specifications, as well as changes in formulation, equipment, process, and site of manufacture are of importance.

In 1994 the FDA established a Working Group to study all aspects in vitro/in vivo correlations for ER drug products. With the completion of the project, a Guidance will be made available that will provide detailed recommendations for developing, validating, and applying correlations for ER products.

3. Dissolution Testing

In general, the conditions as outlined in the 1990 Controlled Release Workshop Report [13] are recommended. This involves testing over the entire range of physiological pH and normally is performed at three or more pH values. Consideration should be given to changing the dissolution medium pH during the test to mimic physiological conditions further. The use of water as the dissolution medium is discouraged. While convenient and simple, water is unbuffered and subject to pH change during testing due to the effects of dissolved drug or excipients, and is considered less likely to provide meaningful data related to the development of in vitro/in vivo correlations.

Where appropriate, different agitation rates (for example, USP basket and paddle methods) should be utilized in the development of a dissolution test procedure. The most

common conditions are paddle at 50 rpm and basket at 100 rpm. However, other agitation speeds should be explored, both to determine the effect of changing agitation rate, if any, on dissolution rate and to attempt to relate dissolution rate to in vivo dissolution/absorption data, where such data are available for the batch of product being tested.

Only aqueous systems seem appropriate, if physiological conditions are desired. While the addition of surfactants is reasonable, forcing higher drug solubility by the use of nonphysiological solvents such as alcohol seems inappropriate. More often than not, in vitro dissolution occurs more rapidly than the corresponding in vivo processes of dissolution and absorption, so the use of nonphysiological media, in general, is not needed. For drugs presenting complex dissolution problems, for example, poorly water-soluble drugs, dissolution procedures that allow media replacement as a means of allowing additional drug to dissolve (such as USP Apparatus 3 or 4: the reciprocating cylinder and flow-through apparatus, respectively) should be considered.

The number of time-sampling points can vary, the goal being to characterize the entire dissolution profile. At least three points are needed to accomplish this: early, middle and late. However, additional, reasonable time points are encouraged.

IV. Bioequivalence Review of Generic Oral Extended-Release Products

For extended-release products, bioequivalence of generic versions of listed drugs is shown by demonstrating that the extent and rate of drug absorption from the generic formulation are not significantly different from the extent and rate of drug absorption from the listed drug. As described in the Guidance [8] above, the Division of Bioequivalence (DBE) requires currently three bioequivalence studies and dissolution data for approval of generic extended-release products.

A. Single-Dose Fasting Study

Study 1 is conducted as a randomized, single-dose, two-way crossover design in healthy adult volunteers under fasting conditions to compare the generic formulation with the reference listed drug. The study objectives are to verify the extended-release nature of the generic product, to ensure that dose-dumping does not occur, and to demonstrate single-dose bioequivalence to the listed drug. Subject selection is discussed in Section V; in general, a sufficient number of subjects must be used to ensure adequate statistical results. More subjects may be required for drugs with high intrasubject variability in the extent or rate of absorption.

Fasting should occur for at least 10 h prior to dosing, and should continue for 4 h postdose. Blood sampling must be frequent enough to define adequately the absorption phase of the plasma concentration-time curve.

The Guidance [8] suggests at least four points on the ascending portion of the curve and six or more points on the descending portion of the curve. The following parameters are reported: area under the curve to the last quantifiable concentration ($AUC_{0\text{-}t}$), area under

the curve extrapolated to infinity (AUCINF), peak concentration (CMAX), and time to peak concentration (TMAX). The log-

transformed values of AUC and CMAX are analyzed as described in another DBE Guidance [19]. Using the two one-sided tests procedure [20], the 90% confidence interval (CI) for the ratio between test (generic) and reference (listed) averages is constructed and should be contained within 80125% of the reference mean. The determination of the terminal elimination rate constant (KEL) and the subsequent calculation of AUCINF are discussed in Section V.

B. Multiple-Dose Fasting Study

Study 2 is a multiple-dose, steady-state, two-way crossover design comparing the generic extended-release product with the listed drug under fasting conditions. The study objective is to demonstrate the bioequivalence of generic and listed products at steady state under fasting conditions. Fasting is observed as in study 1 for extended-release products administered once daily. For products given every 12 h, the morning dose is also given after a 10 h fast; the evening dose is given after fasting at least 2 h and fasting is continued for at least 2 h postdose.

The Guidance [8] states that at least three trough concentrations should be collected on 3 consecutive days at the same time of the day to verify steady-state conditions. Assurance of steady-state conditions is further discussed in Section V. Adequate blood sampling should occur during a dose interval (t) at steady-state to permit determination of AUC_{0-t}, CMAX and TMAX. In addition, fluctuation is calculated in two ways (although the guidance specifies only the first method):

1. Percentage fluctuation [1,8]

% fluctuation = (CMAX - CMIN) * 100 / CMIN

2. Fluctuation index (FI) [1]

FI = (CMAX - CMIN) / (AUC_{0-t} / t)

The log-transformed AUC_{0-t} and CMAX data are subjected to analysis of variance (ANOVA) and their 90% CI values must meet the criteria (80125% of the reference mean) described above.

C. Single-Dose, Three-Way Crossover, Limited Food Study

The objectives of study 3 are to confirm the bioequivalence (using a point estimate of ±20% for AUC and CMAX) of the generic extended-release product to the listed drug, to verify the absence of dose dumping of active ingredient from the generic extended-release product, and to compare the generic product under fed and fasting conditions with regard to labeling information. The study design is a single-dose, three-way crossover with

subjects assigned randomly to all of the six possible dosing sequences. Each subject receives the following treatments:

1. Generic extended-release product after a high-fat meal

2. Designated listed reference product after a high-fat meal

3. Generic extended-release product under fasting conditions

Treatments 1 and 2 are administered 30 min after the meal is begun; the Guidance [8] states that the subjects should be served a high-fat breakfast and then be immediately dosed. Treatment 3 is administered under fasting conditions as in study 1. The standarized meal used currently by the DBE consists of one buttered English muffin, one fried egg, one slice of American cheese, one slice of Canadian bacon, one serving of hash brown potatoes, 240 ml whole milk, and 180 ml orange juice. A comparable food effect occurs if mean values of AUC and CMAX from treatments 1 and 2 are within ± 20%.

D. Dissolution Testing

In the past, the DBE used a dissolution medium consisting of simulated gastric fluid (SGF) without enzyme for 1 h, followed by simulated intestinal fluid (SIF) without enzyme after 1 h [1]. Currently, the DBE requires dissolution testing to be performed over the entire approximate pH range of the gastrointestinal tract, employing distilled water (900 mL) at 37°C at the following constant pH ranges: 11.5, 44.5, 66.5, 77.5. For extended-release capsules, USP 23 apparatus 1 (basket) is recommended at 100 rpm. For extended-release tablets, USP 23 apparatus 2 (paddle) is used at 50 or 75 rpm. Dissolution testing is done for all strengths of the extended-release product using the same lots used in the biostudies. Twelve individual dosage units are tested and the following data reported for every sampling time: percentage released for each unit; mean percentage released; the coefficient of variation for the mean percentage released; and the range of percentage release. The results must adhere to USP 23 Acceptance Table 1.

For extended-release products labeled to be administered every 1224 h, the sampling schedule recommended is: 1 and 2 h, to ensure lack of dose dumping; 4 h, to establish extended-release characteristics; every 2 h, to establish at least 80% release. If release is less than 80%, dissolution conditions should be changed. In general, the range of release at each sampling time should not exceed 30%, with ranges of 2025% preferred by DBE. Specifications for drug release are determined on a case-by-case basis [8].

V. Issues Concerning Bioavailability and Bioequivalence

In addition to discussing types of studies appropriate for ER products, it is useful to consider specific issues related to areas such as study design and evaluation criteria. These issues will be presented in a bioequivalence context, for example,

a study comparing a generic ER product to the innovator ER product, but may be generally applied to bioavailability studies involving ER products as well.

A. Subject Selection

The majority of drug-specific bioequivalence guidances produced by the FDA have recommended the use of healthy adult male volunteers in the age range of 1850 years. Recently, the FDA has issued new guidelines regarding the inclusion of subjects of both genders during clinical trials [21]. The new guidance states that There is no regulatory or scientific basis for routine exclusion of women from bioequivalence trials. Of the reasons for which females were excluded from these studies in the past [22], the potential for variation of pharmacokinetic parameters during a menstrual cycle is acknowledged in the Guidance. This remains a case-by-case issue depending on the literature information available for the drug being studied. The guidance suggests that if such a concern exists, the dosings may be done at the same phase of the cycle. Most studies are two-way crossover designs with washout periods of 1 week. The determination of bioequivalence during this time frame might be susceptible to altered drug clearance due to hormonal variations in the cycle [35]. Therefore, other alternatives might be to use washout periods of 28 days so that all female subjects will be at the same phase of their individual cycles or to exclude women from the study.

B. Stereoisomerism.

FDA has issued a policy statement for the development of new stereoisomeric drugs [23] for IND and NDA submissions. Although no official guidance has been issued by the OGD for ANDA submissions, the determination of bioequivalence for racemic drugs has been discussed [24] and the following guidelines were stated:

1. Generic formulations of innovator chiral drugs must satisfy pharmaceutical equivalence criteria.

2. Chiral composition of excipients may differ between innovator and generic products.

3. The OGD does not wish to impose a requirement for enantiomer-specific assays in the absence of compelling reasons to do so.

4. For drug exhibiting passive absorption, linear active absorption, or poorly extracted drugs with nonlinear postabsorption processes, measurement of the racemate is acceptable.

5. For highly extracted drugs that exhibit postabsorption nonlinear biotransformation, enantiomer-specific assays may be important if nonlinear firstpass metabolism occurs at

labeled doses and affects the two enantiomers differently.

C. Rate of Absorption

The Food, Drug, and Cosmetic Act, as amended by the Drug Price Competition and Patent Term Restoration Act of 1984 [25], requires that, for pharmacokinetic bioequivalence studies, the rate and extent of drug absorption from the generic product not show a significant difference from the rate and extent of absorption of the listed drug. Measurement of rate and extent of absorption also is an essential aspect of bioavailability studies. Although AUC has been accepted as the best measure of extent of absorption, there has been much controversy regarding the proper metric for rate of absorption. Attempts to characterize the absorption phase through compartment-based models using Wagner-Nelson or Loo-Riegelman procedures [26] impose several conditions: (1) the correct model must be known; (2) accurate values of the elimination rate constant must be available from the study; (3) the absorption phase must contain a sufficient number of data points, which is frequently a problem in bioequivalence studies; (4) the absorption rate contant that may be obtained from these procedures may be inappropriate for rate comparisons, since this parameter is scale-independent [27], affecting only the shape and not the magnitude of the curve. The partial area method [27] has also been investigated but with little success. The parameter CMAX/AUC [28,29] has been proposed as a metric superior to CMAX (see below) since it is independent of the amount of drug absorbed and the relative bioavailabilities of generic and listed drugs.

Currently, CMAX is a parameter used by the FDA in the evaluation of bioequivalence, with the 90% CI for the mean ratio required to be within the 80125% range. However, it has been stated that CMAX is not a suitable parameter for comparing rates of absorption since it is confounded with the extent of absorption [28]. The parameter TMAX is also considered qualitatively although no specific criteria have been adopted. The TMAX value depends on both the rates of absorption and elimination of a drug and is independent of extent of absorption [30]. TMAX is a discrete variable dependent on sampling schedule and may require some type of nonparametric analysis for meaningful comparisons of rates; however, no such methods have been adopted by the Agency.

Recently, Bois et al. [31] investigated several metrics as indicators of the rate of absorption. In general, all the measures failed in one way or another, demonstrating inability to detect 25% changes in rate. The authors concluded that rate may not be so important for many drugs, but extent and peak plasma levels are usually major concerns. In addition, variability in absorption rate may exceed 25% even with the same formulation; no currently available metric of rate is sensitive enough to detect a 25% difference in the absorption rate constant. Therefore, the use of CMAX as an indirect measure of rate seems to be the best choice at present.

Finally, it should be noted that the work cited above [22] included simulations using zero-order rate constants that may more closely resemble the properties of extended-release products.

D. Determination of the Terminal Elimination Rate Constant

For the single-dose bioequivalence study (study 1) described above, the parameter AUCINF is determined noncompartmentally by addition of AUC_{0-t} and the factor C_{LAST}/λ_z where CLAST is the last nonzero concentration and λ_z is the terminal phase elimination rate constant. For immediate-release products, λ_z is determined by linear regression of the last several points of the log-linear phase. In the absence of a flip-flop phenomenon, this λ_z value should be similar to the elimination rate constant after intravenous dosing. For an extended-release product, determination of λ_z may give smaller values due to continuous residual drug absorption. In theory, if sampling is continued long enough, all drug absorption will cease due to complete release from the dosage form or excretion of the dosage form from the body. In practice, this may not be achieved, and the λ_z values will be hybrid values resulting from both elimination and absorption. In these cases, the parameter AUCINF should be regarded with caution since the observed λ_z value is not the true terminal elimination rate constant.

E. Determination of Steady State

For a multiple-dose bioequivalence study, the AUC and CMAX during a dosing interval must be determined under steady-state conditions. The guidance [8] states that trough concentrations (CMIN) should be obtained on at least three consecutive days and that these concentrations should be comparable. Since no criteria have been adopted by the FDA, the following are possibilities for verification of steady state.

1. Use of Individual or Population Half-Life

Steady state is defined as the condition in which the rate of drug input equals the rate of drug output, so that drug concentrations in plasma and tissue compartments remain essentially constant. The time required to attain steady-state plasma concentrations is shown mathematically to be dependent on the drug's half-life. After dosing at regularly scheduled intervals (typically, 1224 h for extended-release products) for seven drug half-lives, over 99% of the target steady-state concentration (C_{ss}) is attained. For drugs with intermediate half-lives (≈ 3-12 h) [32], which are likely candidates for extended-release products, 23 days of dosing should ensure steady-state conditions.

If bioequivalence is to be assessed under steady-state conditions, two principal study designs with regard to the washout period might be used. In the first

design (no washout), the first treatment was initiated, dosed to attain steady-state conditions, and intensive blood sampling then occurred over a dosing interval. After sampling was completed, the dosing was continued immediately with the second treatment, allowing the same number of doses to be administered as with the first treatment before sampling began, based on the theory that steady-state conditions would be maintained. With this design, individual half-lives could not be obtained, and population half-life values were used to argue for the attainment of steady-state.

The multiple-dose study design in the current Guidance [8] allows for a washout period between treatments and sufficient sampling so that each subject's half-life after both treatments can be determined. Note that the same limitations as described above for estimation of l_z for an extended-release product apply here. Since the calculated half-life may represent a hybrid value longer than the half-life after administration of an immediate-release dosage form, the assertion of steady state should not be affected.

2. Linear Regression of CMIN Values

Trough values at steady-state (CMIN) are obtained on a minimum of 3 consecutive days at the same time each day. Some sponsors have attempted linear regression of the three or more concentration-time data pairs to argue for attainment of steady state. A slope not significantly different from zero might signify that the CMIN values are relatively constant and indicative of steady state.

The obvious difficulty with this procedure is that only three points may be available for regression analysis, and the accuracy of the determined slope is questionable. Due to variability of individual CMIN values, the slopes may appear to argue against steady-state conditions with either increasing or decreasing values. With four or more points, the procedure may be of some value. The DBE usually requests that this analysis be submitted in order to examine the data for any subjects excluded for not having attained steady state.

3. Comparison of Treatment Means of CMIN Values

One might attempt to demonstrate steady-state conditions by comparing the mean CMIN values from each day to each other using various types of statistical models, including models appropriate for replicate designs. If the TRT*DAY interaction term is statistically significant, the mean CMIN values from each treatment must be compared separately with each other. Although this procedure may be used to determine if the mean CMIN values between any two days are significantly different from each other (at some level of confidence), it does not give any information as to whether any specific subject-treatment combination is at steady state. Therefore, this approach is of very limited value.

F. Limited Food Studies

For generic ER products, a study that compares the extent and rate of absorption of the active ingredient from an extended-release dosage form after a high-fat content meal is required. For innovator ER products, bioequivalence under fasting and food conditions is not a requirement. The primary purpose of such a food study is to define the effect of meals on the release characteristics of drug from the ER products. The following discussion pertains to the bioequivalence study involving food and fasting conditions, for generic ER products.

1. Meal Composition and Timing in Relation to Dosing

The Division of Biopharmaceutics (DOB) and DBE curerntly use different meal compositions in food studies since different objectives are intended. The DOB meal has a higher fat and total caloric content, with the intention of producing a maximal stress condition for the formulation. The DBE meal may more closely resemble a typical breakfast, which is desired for determination of bioequivalence. The optimal meal composition and timing of the dose in relation to the meal may be subjects for future FDA research studies.

2. Study Design

The study design for bioequivalence assessment is a three-way crossover with three treatments and periods, and six sequences. A crossover design with only three sequences is equally efficient at estimating the difference between treatments but is less efficient for evaluation of possible carryover effects [33]. The six-sequence design is balanced for first-order residual effects in that every treatment precedes every other treatment an equal number of times. This type of carryover effect may be considered in relation to the method of data analysis from this study as described next.

3. Data Analysis.

Although not explicitly required by the guidance [8], it may be necessary to subject the log-transformed data from the single-dose food study for extended-release products to analysis of variance (ANOVA) appropriate for a three-way crossover design. Since it is usually not possible to have replacement subjects for each sequence, and there is a greater chance of dropouts over the three periods, many of these studies are unbalanced. It is critical that the most accurate estimate of treatment differences be obtained through ANOVA when an unbalanced study is analyzed.

4. Bioequivalence Criteria

At present the acceptance criteria for the limited food study are stated in the Guidance [8] as generally less than a ± 20% difference between the mean parameter values for test and references products. However, in the case of an unbalanced study design, analysis of the log-transformed data may be more

appropriate. Using the GLM (General Linear Models) procedure of SAS [34], the ESTIMATE statement is used to generate an estimate of the difference between test and reference treatments. This estimate is the logarithm of the test/reference ratio of means. Therefore, the antilog of the estimate is the test/reference ratio of least-squares geometric means. This ratio should be between 0.8 and 1.2 for bioequivalence to be declared.

VI. Scale-Up and Postapproval Changes (SUPAC) for Oral Extended-Release Products: Current and Proposed Regulatory Requirements

A. Perspectives from the New Drug Approval Process

Since 1991, there have been three Workshops cosponsored by the FDA, USP, and AAPS related to various types of changes that may occur for oral immediate-release, oral extended-release, and topical semisolid dosage forms. While many types of changes can occur for both the drug substance and dosage form, those workshops focused on what might be thought of as four important areas, all for the dosage form: formulation, equipment, manufacturing process, and site of manufacture. This covers virtually all changes that may occur directly related to the preparation of a pharmaceutical dosage form. For drug substance, in the ER Workshop Report [6] brief mention of the importance of particle size, surface area, and intrinsic dissolution is made. It is the intention of the FDA to prepare and issue Guidances dealing with these three classes of dosage forms, using the Workshop Reports [6,7,36] as an important source of information. These FDA Guidances will serve as interpretations of current regulations [37] that relate to reporting requirements for these types of changes. At this time, only the IR Guidance [38] has been drafted and released for comment. Based on this draft IR Guidance, the ER dosage forms Workshop Report, and current FDA policies, it is possible to discuss principles related to these areas of change in a somewhat general sense. The final statement of regulatory requirements will be made in the future FDA Guidance.

Other important areas, which do not fall within the scope of this chapter, are chemistry requirements and NDA reporting requirements (e.g., supplement or annual report), will not be covered in this discussion.

1. Formulation Changes

In both the IR and ER Workshop Reports, a table of changes in formulation which may be considered minor was included. While the table is the same in both reports, the ER Report restricts the application of the limits to noncritical components. (Table 2). The report suggests, for those components critical to the release mechanism for an ER product, that all changes should be considered major and the compositional range of these excipients must be based on an in

Table 2 Allowable Range of Noncritical Components (Percentage of Total Formula)

Noncritical Component	% of Total Formula
Filter	5
Disintegrant	
Starch	3
Other	1
Binder	0.5
Lubricant	
Ca or Mg stearate	0.25
Other	1
Glidant	
Talc	1
Other	0.1
Film coat	1

vitro/in vivo correlation. Also, all qualitative changes in formulation, except for deletion of a color, are considered to be major changes.

Regarding biopharmaceutics requirements, minor changes are justified with comparable dissolution data for the current and the proposed revised formulation. Major changes necessitate determination of impact on in vivo release characteristics by way of a bioequivalence study. An in vitro/in vivo correlation may be used in place of a bioequivalence study to justify such changes, if the correlation has been validated with regard to the proposed changes.

2. Equipment

Most equipment changes are considered minor from a biopharmaceutics view-point, if no other changes are involved. If the formulation and manufacturing process are unchanged, dissolution profiles, which show the same release characteristics for old and new equipment, would be all that is normally required.

3. Manufacturing Process

Because of the large number of possible changes, it is difficult to describe precisely every major change in manufacturing process. It is important to state that some changes in manufacturing process are considered major and would require a bioequivalency study if implemented. The Workshop Report [6] is helpful in this regard in stating that the unit operations involved in the manufacturing process must not change for manufacturing process changes to be considered minor. The basic steps for milling, blending,

granulating, drying, or coating currently being utilized cannot be modified to any significant degree for a pro-

cessing change to be considered minor. For example, a process change from direct compression to wet granulation is a major change. It is difficult to describe processing changes any more specifically than this.

4. Site of Manufacture

Because of limited confidence in uncorrelated dissolution testing, a bioequivalence study is required for changes in manufacturing site for ER products. Many of the uncertainties related to this significance of change in equipment and manufacturing process can be addressed if a validated in vitro/in vivo correlation has been established. Such a correlation also will be applicable to changes in manufacturing site and to many formulation changes. In all these situations, comparable dissolution profiles, in lieu of bioequivalency testing, would be all that is required. This emphasizes the desirability of establishing an IVIVC early in the drug approval process.

B. Perspectives from the Generic Drug Approval Process

After an application is approved, manufacturing scale-up to full production batch sizes is permitted as described in the OGD's Policy and Procedure Guide (PPG) 2290 [9]. The size of the test batch, or biobatch, is required as either a net yield of 100,000 finished units or 10% of the maximum production size batch, whichever is greater. Therefore, a maximum 10-fold scale-up may occur post-approval with no additional requirements as long as compliance with PPG2290 is maintained.

Postapproval modification of extended-release products may occur in several ways: manufacturing site change, formulation changes that may or may not involve the release-controlling excipient or mechanism, changes in the manufacturing process or equipment, or scale-up beyond the allowable level. For oral extended-release products, the DBE has generally required a supporting biostudy or studies. (Note: One exception is for changes in a coating, such as a film coating that is not part of the release-controlling mechanism; *in vivo* studies may be waived based on *in vitro* data.) The Workshop Report [6] for scale-up of extended-release products proposed descriptions for minor and major postapproval changes, including both compositional and manufacturing variables.

As of this writing, the OGD/DBE has not issued any guidance for postapproval modifications of extended-release products. Some possibilities have been advanced [39]:

1. For postapproval modifications deemed as *minor* changes, either a confirmatory *in vivo* bioequivalence study or *in vitro* dissolution data might be used to confirm continuing bioequivalence of an extended-release product. The confirmatory biostudy could be done with a minimum of 12 subjects as a single-dose, two-way crossover under fasting

conditions, comparing the

modified generic product with the listed reference product. The dissolution alternative would be through development and validation of a level A correlation as described above.

2. Postapproval modifications deemed as *major* changes would require one or more of the full-sized *in vivo* bioequivalence studies as described previously.

References

1. S.V. Dighe and W.P. Adams. Bioavailability and bioequivalence of oral controlled-release products: a regulatory perspective. In Pharmacokinetics: Regulatory, Industrial, Academic Perspectives. Edited by P.G. Welling and F.L.S. Tse. Marcel Dekker, New York, pp. 307336.

2. USP Subcommittee on Biopharmaceutics. In-vitro/in-vivo correlation for extended-release oral dosage forms. Pharmacopeial Forum 14:41604161, 1988.

3. J.P. Skelly, G.L. Amidon, W.H. Barr, et al. *In vitro* and *in vivo* testing and correlation for oral controlled/modified-release dosage forms. Pharm. Res. 7:975982, 1990.

4. USP Subcommittee on Dissolution and Bioavailability. In-vitro and in-vivo evaluation of dosage forms. Pharmacopeial Forum, 22222234, 1991.

5. USP/HPB/FDA international open conference on dissolution, bioavailability, and bioequivalence. Toronto, Canada, June, 1992.

6. J.P. Skelly, G.A. Buskirk, H.M. Arbit, et al. Workshop II report: scale-up of oral extended release dosage forms. Eur. J. Pharm. Biopharm. 39:162167, 1993.

7. J.P. Skelly, G.A. Buskirk, D.R. Savello, et al. Workshop report: scaleup of immediate release oral solid dosage forms. Pharm. Res. 10:313316, 1993.

8. S.V. Dighe and R.L. Williams. Guidance for oral extended (controlled) release dosage forms: *in vivo* bioequivalence and *in vitro* dissolution testing. 9/9/93. Division of Bioequivalence/Office of Generic Drugs, Center for Drug Evaluation and Research, Food and Drug Administration.

9. D.B. Burlington. Policy and procedure guide #2290: Interim policy on exceptions to the batch-size and production condition requirements for non-antibiotic, solid, oral dosage form drug products supporting proposed ANDA's. Revised, 9/13/90. Office of Generic Drugs, Center for Drug Evaluation and Research, Food and Drug Administration.

10. Code of Federal Regulations 21, 320.25.

11. J.P. Skelly and W.H. Barr. Regulatory Assessment. In Controlled Drug Delivery, Fundamentals and Application. Edited by J.R. Robinson and V.H.L. Lee. Marcel Dekker, New York: 1987, pp. 293333.

12. A. Karin. Importance of assessing food effects in evaluating controlled release formulations. In Oral Sustained Release Formulations, Design and Evaluation. Edited by A. Yacobi and E. Holperin-Wolega. Pergamon Press, New York, pp. 157181, 1988.

13. J.P. Skelly, G.L. Amidon, W.H. Barr et al. Report of the Workshop on In Vitro and In Vivo Testing and Correlation for Oral Controlled/Modified-Release Dosage Forms. J. Pharm. Sci. 70:849854, 1990.

14. J.P. Skelly, W.H. Barr, L.Z. Benet et al. Report of the Workshop on Controlled-Release Dosage Forms: issues and controversies. Pharm. Res. 4:7577, 1987.

15. United States Pharmacopeia 23. In Vitro and In Vivo Evaluation of Dosage Forms. United States Pharmacopeial Convention, Inc.: Rockville, MD, pp. 19241929, 1995.

16. New FDA Initiatives on In Vivo/In Vitro Correlations. 33rd Annual International Industries Pharmacy Conference. Barton Creek, Texas, February 2124, 1994.

17. B. Efron, and G. Gong. A leisurely look at the bootstrap, the jackknife, and cross-validation. Am. Stat. 37:3648, 1983.

18. B. Efron. The jackknife, the boostrap and other resampling plans. Society of Industrial and Applied Mathematics, Philadelphia, CBMS-IVSF Monograph 38, 1982.

19. M.L. Chen, R.N. Patnaik, S.V. Dighe, and R.L. Williams. Guidance for statistical procedures for bioequivalence studies using a standard two-treatment crossover design. Division of Bioequivalence/Office of Generic Drugs, Center for Drug Evaluation and Research, Food and Drug Administration, July, 1992.

20. D.J. Schuirmann. A comparison of the two one-sided tests procedure and the power approach for assessing the equivalence of average bioavailability. J. Biopharm. Pharmacokinet. 15:657680, 1987.

21. Department of Health and Human Services, Food and Drug Administration. Guideline for the study and evaluation of gender differences in the clinical evaluation of drugs; notice. Fed. Reg. 58:39410, 1993.

22. J.D. Henderson, S.V. Dighe, and R.L. Williams. Subject selection and maintenance in bioequivalence studies. Clin. Res. Pract. Drug Regul. Affairs 9:7188, 1992.

23. Department of Health and Human Services, Food and Drug Administration. FDA's policy statement for the development of new stereoisomeric drugs; availability. Fed. Reg. 57:22249, 1992.

24. S.G. Nerurkar, S.V. Dighe, and R.L. Williams. Bioequivalence of racemic drugs. J. Clin. Pharmacol. 32:935943, 1992.

25. Department of Health and Human Services, Food and Drug Administration. Federal Food, Drug, and Cosmetic Act, as amended, Drug Price Competition and Patent Term Restoration Act of 1984, section 505(j)(7)(B), Public law 98417.

26. M. Gibaldi, and D. Perrier. Pharmacokinetics, 2nd ed. Marcel Dekker, New York, pp. 145198, 1982.

27. M.L. Chen. An alternative approach for assessment of rate of absorption in bioequivalence studies. Pharm. Res. 9:13801385, 1992.

28. L. Endrenyi, S. Fritsch, and W. Yan. CMAX/AUC is a clearer measure than CMAX for absorption rates in investigations of bioequivalence. Int. J. Clin. Pharmacol. Ther. Toxicol. 29:394399, 1991.

29. R. Schall, and H.G. Luus. Comparison of absorption rates in bioequivalence studies of immediate release drug formulations. Int. J. Clin. Pharmacol. Ther. Toxicol. 30:153159, 1992.

30. L.F. Lacey, O.N. Keene, C. Duquesnoy and A. Bye. Evaluation of different indirect measures of rate of drug absorption in comparative pharmacokinetic studies. J. Pharm. Sci. 83:212215, 1994.

31. F.Y. Bois, T.N. Tozer, W.W. Hauck, M.L. Chen, R. Patnaik, and R.L. Williams. Bioequivalence: performance of several measures of rate of absorption. Pharm. Res. 11:966974, 1994.

32. M. Rowland, and T.N. Tozer. Clinical Pharmacokinetics: Concepts and Applications, 2nd ed. Lea & Febiger, Philadelphia, pp. 9596, 1989.

33. B. Jones and M.G. Kenward. Design and Analysis of Cross-Over Trials. Chapman and Hall, New York, pp. 189195, 1989.

34. SAS Institute Inc. The GLM procedure. SAS/STAT User's Guide release 6.03 edition. SAS Institute Inc., Cary, NC, pp. 549640, 1988.

35. K. Wilson. Sex-related differences in drug disposition in man. Clin. Pharmacokinet. 9:189202, 1984.

36. G.A. Van Buskirk, V.P. Shah, D. Adair, et al. Workshop III Report: scaleup of liquid and semisolid disperse systems. Pharm. Res. 11:12161220, 1994.

37. Code of Federal Regulations 21, 314.70.

38. Draft Interim Guidance for Immediate Release Solid Oral Dosage Forms: Pre- and Post-Approval changes. November, 1994. Center for Drug Evaluation and Research, Food and Drug Administration.

39. J.D. Henderson, and R.L. Williams. Scale-up and post-approval requirements. Second International Conference on Oral Controlled-Release Dosage Forms. Technomics Publishing Company, Zurich, Switzerland, 1993.

16
Statistical Considerations for Bioavailability/Bioequivalence Studies.

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I. Introduction

To demonstrate that a new formulation can be used as a substitute for an existing formulation, similarities in the efficacy and safety profiles between the new and old formulations have to be established. Because it is difficult and costly to establish the equivalence of clinical endpoints, pharmacokinetic parameters are often used as surrogates. It is important to note that this approach assumes that there is a correlation between the pharmacokinetic parameters and clinical end-points; however, this correlation is often not established.

Bioavailability is defined by the United States Food and Drug Administration [1] as the rate and extent to which the active drug ingredient or therapeutic moiety is absorbed from a drug product and becomes available at the site of drug action. Because it is often difficult to measure drug concentrations at the site of action, drug concentration in the systemic circulation (e.g., blood) is used as a surrogate.

There are a number of different types of bioavailability studies. In this chapter we will focus the discussion on comparative bioavailability studies, that is, bioavailability studies that are used to assess the equivalency of the rate and extent between two (or more) formulations. Other pharmacokinetic studies such as food effect, age effect, and dose proportionality studies can also be designed and analyzed using the same concepts.

In bioavailability/bioequivalence studies, regulatory agencies often require the sponsor to collect blood and urine samples at specific time points from healthy volunteers in a randomized, controlled, open-label study [2]. Drug concentrations are determined from these samples to calculate pharmacokinetic parameters used to estimate the rate and extent of absorption. If the difference in rate and extent of absorption between two formulations is small, one may conclude that the formulations are bioequivalent.

The objective of this chapter is to provide an overview of the statistical issues associated with bioavailability/bioequivalence studies. For a general discussion about pharmacokinetic issues, the reader is referred to the chapter by Weiner and Yuh [3]. III, Pharmacokinetic parameters most often used to assess bioavailability are discussed in Section II. In Section III, designs of bioavailability studies, including parallel designs, crossover designs with repeated treatments, and multitreatment, partially balanced, incomplete crossover designs, are reviewed. Analysis strategies for bioavailability studies are provided in Section IV as well as specific acceptance criteria for both population bioequivalence and individual bioequivalence. Finally, a number of statistical issues requiring further intensive research are presented in Section V.

II. Response Variables

Blood or urine samples are frequently collected at specific time points to assess the concentration-time profile. Depending on the dosage form, primary param-

eters in bioavailability studies often include those discussed in the following sections.

A. Area Under the Concentration Curve

Area under the plasma concentration-time curve (AUC) is used to measure extent (total amount) of drug absorbed. AUC[0-tldc], AUC from time zero until the time of the last detectable concentration, may be calculated using the linear trapezoidal rule:

$$AUC_{[0-T]} = \sum_{i=1}^{N} \frac{[C(t_{i-1})]}{2} (t_i - t_{i-1}), \quad (1)$$

where $t0 = 0$ and $T = tN$ = last time point.

To avoid dependence on time of last sampling, the AUC is extrapolated to infinite time, $AUC_{[0-\infty]}$, by adding the term C(T)/l to Equation (1), where l represents the terminal elimination-rate constant, which is estimated as the absolute value of the slope of a simple linear regression of the natural logarithm of the plasma concentration during the terminal phase of the plasma concentration-time profile. If l can not be precisely estimated, it is preferable to report AUC[o-tldc] [4].

In a multiple-dosage study, $AUC(0\text{-}_t)$ (where t is the dosing interval), is the AUC of interest.

B. Maximum Plasma Concentration

The maximum observed plasma concentration (C_{max}) is often used to assess the rate of drug absorption. The observed peak concentration may not represent the true peak response and is more variable than AUC since the samples are collected at discrete time points. Also, C_{max} depends on the extent as well as the rate of absorption.

In addition to the primary parameters, there are a number of secondary parameters.

C. Time to Maximum Plasma Concentration

The observed time to maximum plasma concentration (t_{max}) in used jointly with C_{max} to measure the rate of absorption. Because of the limited number of time points used in the study, in general, t_{max} is much more variable than AUC and C_{max}.

D. Elimination Half-Life

The elimination half-life of a drug can be derived by dividing 0.693 by the absolute value of the slope of the terminal linear phase of the concentration-time profile presented on semilog scale.

Other parameters such as the estimated absorption rate and concentration-time profiles are also used to characterize bioavailability [5]. However, they are not routinely reported in bioequivalency trials. For studies collecting urine samples, the cumulative percentage of drug recovered, which is calculated as the cumulative amount recovered in some time interval divided by the initial dose, is often used as the primary parameter.

In a multiple-dosage study, both single-dosage and steady-state pharmacokinetic parameters are used to assess bioavailability. Steady-state AUC and C_{max} often serve as the primary parameters. Note that the steady-state minimum plasma concentration (C_{min}) and the fluctuation index [(C_{max} - C_{min})/C_{avg}], where C_{avg} is the average concentration across the time points [AUC(0-t)/t, where t is the dosing interval], are also parameters of interest in a multiple-dosage study.

Since the samples are collected at finite time points and parameters are estimated from individuals, except AUC and C_{max}, the variability of the estimated pharmacokinetic parameter is often large [6]. In particular, since the fluctuation index is the ratio of two pharmacokinetic parameters, it may be necessary to apply an appropriate transformation to obtain a more normal-like distribution.

III. Study Design

The variability within individual subjects is generally higher than the variability between the subjects in bioavailability/bioequivalency studies. Thus, unless the drug has an extremely long half-life (in which case carryover effects may occur) or toxicity problems, crossover designs are often used to assess the formulation effect.

For two formulation bioequivalence studies, a two-period, two-sequence crossover design is often used to assess bioequivalency (Table 1). Letters are used to represent treatment groups. It is extremely important to have an appropriate washout period in crossover studies. Usually, it requires at least five times the terminal half-life of the drug.

A three-period, six-sequence crossover design is presented in Table 2, and a four-period, four-sequence crossover design is shown in Table 3. They are used

Table 1 A Two-Period Crossover Design

		Period	
Sequence		1	2
	1	A	B
	2	B	A

Table 2 A Three-Period, Six-Sequence Crossover Design

	Period		
Sequence	1	2	3
1	A	B	C
2	B	C	A
3	C	A	B
4	B	A	C
5	C	B	A
6	A	C	B

Table 3 A Four-Period, Four-Sequence Crossover Design

	Period			
Sequence	1	2	3	4
1	A	B	C	D
2	B	D	A	C
3	C	A	D	B
4	D	C	B	A

to assess formulation differences for studies with three and four treatment groups, respectively. These designs are not exactly equivalent to Latin square designs because the order of periods is fixed. All designs mentioned above are balanced because the number of occurrences of each formulation in each period is the same, and the occurrence of all comparisons in a common sequence is the same.

Formulation effects can be assessed using the within subject variability in a crossover design. Thus, with fewer subjects, the estimate of the treatment effects can be more precise than that obtained from a parallel design. However, in a two-period, two-sequence crossover design, the carryover effects are confounded with the treatment, sequence, and period effects and cannot be estimated independently. Thus, it is difficult to conclude bioequivalency with the presence of both sequence and period effects. Note that treatment effects can be estimated independently of carryover effects in the three-period, six-sequence and four-period, four-sequence crossover designs.

When there are a large number of formulations, a complete crossover (all subjects receive all treatments) study may be difficult to conduct because of the potentially higher dropout rates and carryover effects. These studies may also

require many more blood samples. In addition, such studies are more time-consuming to perform. Westlake [7] has discussed the use of balanced incomplete block designs to compare a large number of treatment groups. Yuh and Ruberg [8] also suggested similar approaches in a drug to drug interaction study. Since the primary comparisons of interest are not the same as all possible treatment comparisons in their study, Yuh and Ruberg presented a number of balanced or partially balanced incomplete crossover designs and a cluster of complete crossover designs using an oversampling technique to assess the treatment effects. One of their proposed designs is shown in Table 4.

As mentioned earlier, the carryover effect is confounded with the formulation, sequence, and period effects in a two-period, two-sequence crossover design. An extended period design (e.g., the Balaam design) can be used to assess the true formulation difference. These extended period designs usually are crossover designs with extra periods and repeated treatments. That is, subjects will receive one of the treatments at least twice. A two-treatment, three-period, two-sequence extended period design is presented in Table 5. Extended period designs also provide a good estimate of the formulation by subject interaction [911]. Recently, individual relative bioavailability has been a popular topic of research

Table 4 A Balanced Four-Period, Seven-Sequence Crossover Design

	Period			
Sequence	1	2	3	4
1	A	A+C	C	A+B
2	B	B+C	A+B	A+C
3	C	A+B+C	A+C	B+C
4	A+B	A	B+C	A+B+C
5	A+C	B	A+B+C	A
6	B+C	C	A	B
7	A+B+C	A+B	B	C

Table 5 An Extended Period Crossover Design

	Period		
Sequence	1	2	3
1	A	B	A
2	B	A	B

interest. Extended period designs can be used to assess individual bioequivalence. A more detailed discussion will be provided in Section IV.

After an appropriate design has been identified, a sample size estimate must be obtained. The methods to derive the number of subjects required depend on the bioequivalence criterion. As an example, a procedure using the current FDA bioequivalence criterion for the solid dosage form will be used to demonstrate how one can obtain an estimated sample size. This criterion is established based on the ratio of treatment averages. For a given within-subject coefficient of variation, which may be obtained from the results of analysis of variance in a pilot study or a publication, the sample size is chosen such that there is a sufficient high probability (e.g., 80%) to ensure that the 90% confidence limits for the ratio of treatment averages will be within 80120% (or 80125% if a log transformation is performed). Phillips [12] provided a procedure incorporating the noncentrality parameter (the difference between the test and reference formulations) to estimate the sample size. For example, if the coefficient of variation is 20% and the mean difference is 5%, a study with 24 subjects will be required to ensure that the end points of the 90% confidence interval for the ratio of formulation means are within the 80120% range with an 80% probability.

To avoid a potential food or drug to drug interaction, blood or urine samples should always be drawn just prior to administration of drug. In order to minimize the variability of the estimated parameters, appropriate sampling time points for collecting blood or urine samples have to be determined. For oral dosage forms, it is obvious that samples should be taken more frequently during the absorption phase when the concentration-time profile is changing quickly and less frequently during the elimination phase. However, if the objective is to analyze $AUC_{[0-\infty]}$, it is necessary to allocate suitable time points in the terminal phase to obtain a reliable estimate for t½ and $AUC_{[0-\infty]}$.

A number of statistical approaches have been presented to determine the optimal sampling time. We will refer to the papers by D'Argenio, Distfano, Landaw, Katz and D'Argenio, and Drusano et al. [1318].

IV. Analysis

A. Population-Relative Bioavailability

Population-relative bioavailability is defined as the ratio of the average test formulation effect over the average reference formulation effect.

Since most bioequivalence studies use crossover designs, the crossover analysis of variance model is often utilized to assess the formulation effects. That is, one often uses

the following statistical model to assess bioequivalence:

$$X = \mu + SEQ + SUB(SEQ) + PER + FORM + \epsilon$$

where X = parameter of interest, μ = overall mean, SEQ = sequence effect, SUB(SEQ) = subject effect nested within sequence effect (between subject error), PER = period effect, FORM = formulation effect, and ϵ = within-subject error.

The sequence effect is assessed using the between subject error (SUB[SEQ]) while period and formulation effects are assessed using the within-subject error. The estimated sequence effect is less precise than the formulation or period effect because the between-subject variability is usually larger than the within-subject variability for pharmacokinetic variables. For a three-treatment, three-period, six-sequence design; a four-treatment, four-period, four-sequence design; or a Balaam design; carryover effects should be incorporated in the model to assess true formulation differences. Many statistical analysis packages (e.g., SAS®) can be easily programmed to conduct the analysis. However, it may be necessary to use a dummy variable technique to assess the carryover effect.

Traditionally, F-tests are performed and p values are reported to test the significance of period and sequence effects. For the formulation effect, a number of approaches have been proposed and the majority use either the confidence interval or the p value approach.

1. Confidence Interval Approach.

a. Traditional Confidence Interval The goal of traditional hypothesis testing is to attempt to show that there is a difference between the treatment groups. However, the objective of bioequivalency trials is to prove that there is no difference between the treatment groups. One approach is to choose a large significance level to ensure that the test has an adequate power to detect the difference if there is a true difference between treatment groups [19]. However, a paradox may be created because a well-designed study will produce a more precise estimate. Thus one may interpret a small treatment difference as statistically significant for less variable data while declaring bioequivalence for a poorly designed (more variable) study.

Another approach to assess bioequivalence is to use confidence intervals as discussed by Westlake, Metzler, and Shirley [2022]. An approximate confidence interval for the ratio of test and reference means can be derived from the results of an analysis of variance. Specifically, one can construct the confidence interval for the least-squares mean difference between test and reference formulations. If the reference mean is treated as a constant, a simple linear transformation can be performed to obtain an approximate confidence interval for the ratio of least-squares treatment means. Two products are bioequivalent if the end points of the percentage scaled 90% confidence interval for the ratio are within the 80120% range. This rule has incorporated both the mean difference

(the bias) and the variability of the difference in consideration. That is, as long as the end points of the percentage scaled 90% confidence interval are within

the 80120% range, two formulations are bioequivalent even if their means are significantly different ($p < 0.05$).

The percentage scaled 90% confidence interval for the ratio of formulation means is computed as follows:

$$\left\{\left[\frac{\bar{X}_T - \bar{X}_R}{\bar{X}_R} \pm \frac{t_{v,0.95}S_d}{\bar{X}_R}\right] + 1\right\} \times 100\% \quad (2)$$

where $\bar{X}$i = least squares mean observed response with formulation i, i = T,R (where T and R denote the test and reference formulations), S_d = standard error of the least-squares mean difference in formulation means using the mean square error obtained from the analysis of variance, v = degrees of freedom associated with the mean square error.

If the reference formulation can not be treated as a constant, an exact confidence interval for the ratio using Fieller's theorem can be derived [23,24]. Note that the exact confidence interval is usually somewhat wider than the approximate confidence interval.

b. Symmetrical Confidence Interval Because the confidence interval for the ratio is not symmetrical about the origin but the acceptance criteria is symmetrical about the origin, Westlake [25] has modified the classic approach to develop a symmetrical confidence interval. Westlake has indicated that the symmetrical interval is used merely as a decision-making tool. These intervals do not show the location of the sample values and are always wider than the traditional confidence intervals [26]. The confidence coefficient of the symmetric confidence interval is also always greater than 1-a. Furthermore, since the sample mean is used as a constant, the symmetrical confidence interval is only an approximation. The exact symmetrical confidence interval has been derived by Mandallaz and Mau [27]. The reader is referred to the papers by Steinijans and Diletti, Kirkwood, and Metzler [2830] for additional discussion of the statistical properties of the symmetrical confidence interval.

2. Power Approach

Since the objective of bioequivalence studies is to show that the reference and test formulations are similar (i.e., the alternative hypothesis is $\mu_R = \mu_T$), the FDA has defined that two products are bioequivalent only if the p value associated with the formulation effect is larger than a prespecified level (e.g., .05) and there is at least a power of 80% to detect a 20% relative difference between the test and the reference products. This rule is no longer used by the FDA as the primary criterion for assessing bioequivalence [31]. It is of interest that the Japanese government has proposed to report the minimum difference that can be detected at a predetermined sample size and power. A clinical decision is made based on the minimum detectable difference of two formulations.

A two-period, two-sequence crossover study with a washout period of 3 days is used to demonstrate these procedures. The objective is to assess the relative bioavailability of two batches of drug X. Twenty-four healthy subjects were randomly assigned to each of the two sequences. The primary parameter of interest is the area under the concentration-time curve (024 h). The raw data are listed in Table 6. The results of the analysis of variance are presented in Table 7.

Substituting the appropriate values into Equation (2), we obtained 89.4%, 105.9% as the 90% confidence interval for the ratio in formulation means. Because the end points of the percentage scaled confidence interval are within the 80120% range, these formulations are considered bioequivalent. The same conclusion can be reached using either the symmetrical confidence interval or the power approach.

Table 6 Individual AUC Values (µg.hr/ml)

	Period 1		Period 2	
Subject	Formulation 1	AUC	Formulation 2	AUC
1	2	1697.45	1	1636.21
2	1	800.54	2	636.29
3	2	1050.98	1	615.61
4	1	1049.72	2	1113.16
5	2	936.53	1	1133.20
6	1	1504.35	2	1094.96
7	2	1277.87	1	1603.63
8	2	1548.28	1	1411.44
9	2	964.41	1	129.55
10	1	1458.60	2	1341.08
11	1	1602.51	2	1470.85
12	1	688.35	2	576.33
13	1	1841.53	2	1312.68
14	1	1259.93	2	1037.75
15	1	1227.14	2	931.89
16	2	1339.08	1	1241.25
17	2	931.91	1	613.14
18	2	1136.23	1	547.66
19	2	1534.56	1	823.46
20	2	794.44	1	952.24
21	2	1538.59	2	1131.16
22	1	874.06	2	1133.22
23	2	1478.62	1	1385.12
24	1	1328.58	2	1131.90

Table 7 Analysis of Variance for AUC Data

Source	df	Sum of Squares	F
Sequences	1	1878.82	0.01
Subjects (sequences)	22	3869649.05	
Periods	1	9150.34	0.24
Formulations	1	310807.54	8.18
Error	22	836364.11	
Summary of results			
Estimate of $\mu T/\mu R$		97.7%	
90% Confidence interval		(89.4%, 105.9%)	
90% Westlake interval		(90.9%, 109.1%)	
Power (a = 0.05)		0.977	

3. Anderson and Hauck's Procedure

Since type II error is more important in equivalency trials, one may set up an alternative hypothesis that the formulation difference will fall within a predetermined interval. This idea was discussed by Lehmann [32] and Bondy [33]. Anderson and Hauck [34] have adopted this approach using the following pair of hypotheses.

$$H_N: \mu_T - \mu_R \leq \log(0.8) \text{ or } \mu_T - \mu_R \geq \log(1.25)$$

$$H_A: \log(0.8) < \mu_T - \mu_R < \log(1.25).$$

The null hypothesis states that the two formulations are inequivalent and the alternative hypothesis states that they are equivalent.

The test statistic the authors consider is

$$T = \frac{\bar{X}_T - \bar{X}_R - 1/2[\log(0.8) + \log(1.25)]}{S(1/n_T + 1/n_R)}$$

where the Xs and ns are sample means (in the logarithmic scale) and sample sizes, respectively, and S is the standard deviation (square root of mean square error) obtained from the analysis of variance table. The authors have indicated that the underlying distribution of this test is unknown. Thus, the exact critical value can not be found easily. However, Student's distribution can be used as an adequate approximation. A similar approach was proposed by Rocke [35]. There is a small chance that one may conclude that the two formulations are equivalent even if the difference of the two formulations means is not within the acceptable range.

4. Bayesian Approach

Rodda and Davis [36] suggested that one can assess bioequivalency using bayesian analysis. They proposed to calculate the posterior probability that the observed relative bioavailability is within the acceptable limits. One will conclude bioequivalence if this posterior probability is greater than a prespecified level (e.g., .90). Selwyn et al. [37,38] have also developed a more complex statistical model using the bayesian concept. Wijnand and Timmer [39] have shown that Rodda and Davis' rule is equivalent to Westlake's symmetrical confidence interval rule, while the decision rule based on Mandallaz and Mau's confidence interval is equivalent to that proposed by Selwyn et al. Finally, the approach using the Gibbs sampling was examined by Gelfand et al. [40].

5. Two One-Sided Tests Procedure

A procedure that consists of a pair of hypothesis tests was proposed by Schuirmann [41].

$$H_{N1}: \mu_T - \mu_R \leq .8$$

$$H_{A1}: \mu_T - \mu_R > .8$$

and

$$H_{N2}: \mu_T - \mu_R \geq 1.2$$

$$H_{A2}: \mu_T - \mu_R < 1.2$$

This procedure divides the interval hypotheses H_N and H_A into two sets of one-sided tests. This procedure was originally developed based on the log scaled values. That is, the logarithmic transformed mean difference was compared to log(0.8) and log(1.25). This test will conclude bioequivalence of two formulations only if both H_{N2} and H_{N2} are rejected at a chosen nominal level of significance (e.g., 0.05). The decision rule based on the two one-sided tests procedure (a = 0.05) is identical to that based on the traditional 90% confidence interval.

Schuirmann also compared the rejection regions and the probability characteristics of his procedure to the power approach through a simulation exercise and concluded that this procedure is more reasonable than the power approach.

In a simulation study, Metzler [30] evaluated the power of different decision rules: the traditional confidence interval approach (the two one-sided tests procedure), Westlake's symmetrical confidence interval (Rodda and Davis' procedure), Mandallaz and Mau's approach, and Anderson and Hauck's testing procedure. The power of the decision rule for bioequivalence was defined to be the probability of rejecting bioequivalence given a

true ratio of treatment means. The rule based on the symmetrical confidence interval is similar to Anderson and Hauck's procedure if the coefficient of variation (the relative standard deviation) is less than 20%. The probability of rejection is close to 0.90 for all

rules excluding the two one-sided tests procedure. In order to make the rule based on the two one-sided tests procedure more like the others, a larger a should be used. He also indicated that only the rule by Mandallaz and Mau is different from the others if the coefficient of variation is greater than or equal to 30%.

B. Individual-Relative Bioavailability

Because of the nature of crossover designs, test and reference data can be compared within each subject. That is, there is an estimate of relative bioavailability for each subject if there is neither a period nor a sequence effect. Since each ratio measures relative bioavailability for an individual, the central tendency for individual bioavailability (ratios) may be more meaningful than the ratio of the average bioavailability (the means) [4249]. My personal experience with clinicians has indicated that they often interpret relative bioavailability in terms of individual ratios. Thus, to ensure that an individual patient could be switched to a different formulation, it is essential to assess the individual bioavailability [45]. Note that under the logarithmic transformation, the individual bioavailability approach will be identical to the population bioavailability approach because

$$(1/n)\left[\sum_{i=1}^{n}\log(X_{iT}/X_{iR})\right] = (1/n)\left[\sum_{i=1}^{n}\log(X_{iT})\right] - (1/n)\left[\sum_{i=1}^{n}\log(X_{iR})\right],$$

where n = number of subjects in the study.

1. Analyzing Individual Bioavailability as a Continuous Variable

a. 75/75 Rule The FDA [46] designed a rule using the individual ratios. This rule stated that at least 75% of the subjects in the study were required to have their bioavailability ratio within the interval 75125% to claim bioequivalence between two formulations. The concept of this rule is similar to that of the tolerance interval approach. One objective of this rule is to take into account the variability of bioavailability and the correlation between two formulations in addition to the means. However, this arbitrary rule came under criticism, in part, because of its high rejection rates [44,45,4748]. The FDA no longer uses the rule in assessing bioequivalency because it is not statistically valid.

b. Tolerance Interval Approach Another reasonable approach is to compute a parametric or a nonparametric tolerance interval for individual ratios. That is, one can compute a probability that a certain random interval includes (or covers) a preassigned percentage of the probability for the distribution of the individual ratios. Two formulations are equivalent if the 90% tolerance limits for 90% of the individual bioavailability are within 80120% range. To obtain a reasonable width of the tolerance interval, a large sample size is required [50].

c. Yuh's Robust Estimate Approach The distribution of individual ratios is generally either skewed or highly variable even if the pharmacokinetic parameter is normally distributed. Therefore, Yuh [43] proposed a procedure using an M-estimator with a t-distribution (d.f. = 6) weight function, the t-estimate, to assess the central tendency of individual bioavailability. This t-estimate is more robust (efficient) than the sample mean and other traditional nonparametric estimates (e.g., the sample median) for many underlying distributions. One can conclude that two formulations are bioequivalent if the confidence limits of the t-estimate are within the 80120% range. For comparative purposes, it was also shown how to use the exact confidence interval for the sample median of the ratios to assess individual bioequivalence. The reader is referred to the paper by Yuh and Ruberg [44] for more detailed discussion.

2. Analyzing Individual Bioavailability as a Categorical Variable

Individual bioavailability is similar to percentage change from baseline for efficacy parameters in clinical trials. When analyzing clinical efficacy, one approach is to define a responder and analyze the percentage of patients responding to drug (responder rate) to assess treatment effects. This approach inspired Anderson and Hauck [45] to propose analyzing individual bioavailability as a categorical variable. Their decision rule, test of individual equivalence ratios (TIER), is based on comparing the one-sided confidence interval for the proportion of subjects with individual ratios falling within the predetermined acceptable limits (e.g., 80120%) to a specified probability (called MINP, the minimum proportion of the ratios in which the two formulations must be equivalent to call the two formulations [individual] bioequivalent [e.g., MINP = 80%]). Because of the nature of binary response variables, it requires a large sample size to make a meaningful assessment. For example, for studies with samples of size 1421 subjects, all subjects in the study must be bioequivalent in order to conclude the two formulations are bioequivalent if MINP = 80%. Also, this rule may not be valid if sequence and/or period effects exist.

C. Others

In the past few years, many procedures have been developed based on the assumption that the underlying distribution of two formulations is bivariate: in particular, a bivariate normal distribution with mean $\mu = (\mu T, \mu R)$ and covariance matrix.

$$\begin{pmatrix} \omega_T^2 & \omega_{TR} \\ \omega_{TR} & \omega_R^2 \end{pmatrix}$$

The parameters wT and wR are the between-subject variabilities associated with the test and reference formulations. The correlation (r) between the two formulations is r =

$w^{TR}/w^{T}w^{R}$. If one assumes that there is no period or sequence

effect, two formulations are equivalent if the means and variabilities are similar and the correlation is near 1. Methods in the previous sections can be used to compare the means while the Pitman-Mogan test [50] can be used to examine the variability of correlated data. A simple linear regression can be used to assess the correlation. There are other procedures that simultaneously incorporate means, variabilities and the correlation.

1. Concordance Correlation Approach

Yuh [52] suggested combining the mean and variability differences and the correlation into one index, the concordance correlation. Because of the bias associated with other traditional approaches, a reproducibility index was used to assess the agreement between two measurements from the same subject by estimating the variation from the 45 degree line through the origin.

For example, in a two-period crossover study, let Xij be the response from the *i*th subject from the *j*the treatment group, where i = 1, 2,, N, N = number of subjects in treatment j and j = R, T. The Xij are assumed to be independently selected with means μ = (μT, μR) and covariance matrix

$$\begin{pmatrix} \omega_T^2 & \omega_{TR} \\ \omega_{TR} & \omega_R^2 \end{pmatrix}$$

where wT and wR are the between-subject variabilities associated with the test and reference formulations.

It can be shown that

$$E[(XT - XR)2] = (\mu T - \mu R)2 + (wT - wR)2 + 2(1 - r)wTwR,$$

where Xj = (Xij) i=1,2, , n, n = the number of subjects, j = R, T, and r is the Pearson correlation coefficient. The expected value, E(XT - XR)2, represents the expected squared perpendicular deviation from the 45 degree line, multiplied by 2. It is used as a measure of the degree of concordance between the test and reference formulations.

The following transformation is used to scale this value between -1 and 1:

$$\begin{aligned} \rho_c &= 1 - E\left[(X_T - X_R)^2\right] / \omega_T^2 + \omega_R^2 + (\mu_T - \mu_R)^2 \\ &= (2\omega_{TR})/[\omega_T^2 + \omega_R^2 + (\mu_T - \mu_R)^2] + \rho C, \end{aligned}$$

where C = [(n + 1/n + u2/2]-1, n = w*T*/w*R* = scale shift, u = (mT - mR)/ $\mu_R)/\sqrt{\omega_T \omega_R}$ = location shift relative to the scale, and r*c* = the concordance correlation coefficient.

Note that 0 < C ≤ 1 and that C is a measure of both the mean and variability differences. If between-subject variabilities are similar between formulations, and the correlation is near 1, this index is nothing but an estimate for the population equivalence. The

concordance correlation is equal to the Pearson correlation if both means and variabilities are identical. Also, note that $E(X^T -$

$X_R)^2$ is equal to 0 and the concordance correlation coefficient is equal to 1 if the responses to the test and reference products are identical for all subjects. The confidence interval for this index can be obtained using the bootstrap method or the asymptotic standard error. This procedure is easy to compute and considers not only the mean difference but also the variability difference as well as the correlation. However, a regulatory acceptance criterion has not been established for this procedure. A two-period crossover study with eighteen healthy volunteers was used to demonstrate the above methods. This study was conducted to evaluate relative bioavailability of a new formulation of an experimental antihistamine agent [43]. Individual AUC values and ratios are listed in Table 8 and depicted in Figure 1. The concordance correlation coefficient and its confidence interval are presented in Table 9. For the purpose of comparison, the 90% confidence interval for the ratio of the means (the two one-sided test approach), the 90% confidence interval for the median of the ratios, the 90% confidence interval for the robust t-estimate of the ratios, the 90% nonparametric tolerance limits for 90% of the ratios, the Pearson correlation coefficient, and the estimated slope and intercept of the regression line are also listed. The

Table 8 Individual AUC Values (ng.hr/ml) and Ratios

Subject	AUC Reference	AUC Test	Ratio (T/R) (%)
15	4108	1755	43
3	2526	1138	45
9	2779	1613	58
13	3852	2254	59
12	1833	1310	71
8	2463	2120	86
18	2059	1851	90
17	1829	1682	92
2	2594	2613	101
4	2344	2738	117
16	1864	2302	124
6	1022	1284	126
10	2256	3052	135
5	938	1287	137
7	1339	1930	144
14	1262	1964	156
11	1438	2549	177
1	1735	3340	193
Mean	2124	2043	109
± S.D.	± 866	±643	±44

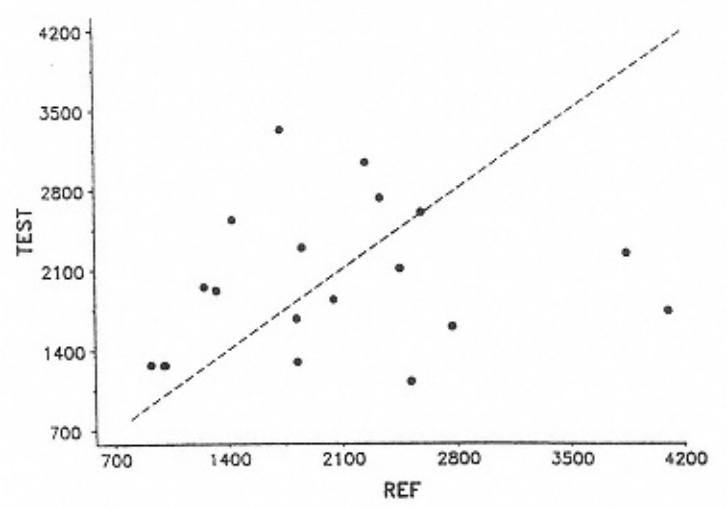

Figure 1
Plot test AUC values versus reference AUC values.

Table 9 90% Confidence/Tolerance Intervals Using Different Procedures

Two one-sided test approach	96% (79%, 113%)
The median of ratios	109% (86%, 135%)
Robust t-estimate of the ratios	108% (91%, 125%)
Tolerance limits	(45%, 177%)
Pearson correlation coefficient	0.11 (-0.22, 0.44)
Linear regression	Slope = 0.08, (-0.237, 0.4) Intercept = 1872 (1146, 2597)
Concordance correlation coefficient	0.10 (-0.18, 0.39)
	$(\overline{X}_T - \overline{X}_R)/\sqrt{S_T S_R} = 0.11\ (-0.44, 0.67)$
	ST/SR = 1.37 (0.82, 2.09)

confidence limits of the median are calculated using the exact confidence limits, and the confidence limits of the Pearson and concordance correlation coefficients are computed using the bootstrap method with 1000 replicates.

It is clear that if one uses a 80120% interval as the acceptance criterion for equivalency, the only procedure that supports the bioequivalence conclusion is the two one-sided tests approach. The concordance correlation is only 0.10. After examining each component of the concordance correlation further, it appears that the variabilities of these products may be somewhat different even if the means are similar. In addition, there is a weak linear correlation between these formulations. Since the traditional methods focus on comparisons of population averages, they can not detect either a significant variability difference or a poor correlation.

2. Relative Risk Approach.

Another way to incorporate the mean difference, the variability difference, and the correlation is to use a likelihood ratio test proposed by Sheiner [53]. Two indices were derived for population and individual bioequivalence, respectively, based on the ratio of the expected squared loss (risk) functions associated with the formulations. Specifically, the relative individual risk (RIL) is defined as

$$RIL = E[(X_T - \mu_R)^2] / E[(X_R - X_{R'})^2]$$

$$= [(\mu_R - \mu_T)^2 + (w_T + w_R - 2w_{TR}) + s_T] / s_R,$$

where X_R and X_T are the responses from the reference and test formulations, respectively, and $X_{R'}$ is the response from the reference formulation taken at a different time point. The parameters μ_R and μ_T are population means associated with the reference and test formulation, respectively, and s_R and s_T are the within-subject variabilities associated with the reference and test formulation, respectively; w_R and w_T are the between-subject variability associated with the reference and test formulations, and w_{RT} is the covariance between two formulations. The second term in the parentheses in the numerator is proportional to the subject by formulation interaction term discussed by Hwang et al. [10] and Ekbohm and Melandar [9]. If within (and between) subject variabilities are similar for the test and reference formulations and the correlation is near 1, this index is nothing but an estimate for the population equivalence.

Using an extended period crossover design, the maximum likelihood estimates for (μ_T, μ_R, s_T, s_R, w_T, w_R, w_{TR}) can be obtained under the normality assumption and the absence of period and sequence effects. For hypothesis testing, one may reject bioequivalence if $RIL > RIL^*$, RIL^* is a preassigned value, at a = 0.05.

3. Absolute Risk Approach

Instead of taking the ratio of the expected mean squared errors, Schall and Luus [54] developed a similar procedure using the difference of the expected mean

squared risks. Using the same notations as in Section IV.C.2, bioequivalence was defined in terms of the difference of

$$E[X_T - E(X_R)]_2 \text{ and } E[X_R - E(X_{R'})]_2.$$

$$\text{Note that } E[X_T - E(X_R)]_2 - E[X_R - E(X_{R'})]_2$$

$$= (\mu_T - \mu_R)_2 + w_T + w_R - 2w_{RT} + s_T - s_R$$

$$= Q.$$

Two formulations are not similar if this quantity is large. A nonparametric bootstrap algorithm was used to obtain a one-sided confidence interval for this quantity using the percentiled method. Bioequivalence is declared if the 100(1 - a) percentage lower limit for Q is greater than or equal to a preassigned value. Note that this algorithm can be modified if there is a period effect.

A test for population equivalence using similar principles was also developed. The relationship of the absolute risk approach to the proposal of Anderson and Hauck [45] was discussed and argued that the proposal by Anderson and Hauck is a special case of the absolute risk approach.

Bioequivalence cannot be evaluated using the absolute risk approach with data from a regular two-period, two-sequence crossover study. Again, similar to the relative risk approach, an extended period design is required for the employment of these rules.

V. Discussion and Conclusion

Because C_{max} is confounded by the extent of absorption and t_{max} is not very precisely measured, a number of new estimators of the rate of absorption have been proposed. In particular, the area under the curve from time 0 to t_{max}, $AUC[0, t_{max}]$ was discussed by Chen [55] and Tozer [56] and C_{max}/AUC was analyzed by Lacey et al. [57]. In addition, moment analysis was utilized to compare C_{max}, t_{max}, and mean absorption time (MAT) [5]. There is clearly a need to investigate these parameters thoroughly.

A number of designs for bioavailability trials have been reviewed in this chapter. In order to compare both the average bioavailability and the variability, it is necessary to utilize extended period crossover designs. These designs also provide an estimate of the correlation of the formulations. However, they are time-consuming and add additional cost. Higher dropout rates and more pharmacokinetic samples are also expected from these designs. For drug products that have a narrow therapeutic window (e.g., the anticonvulsant drug, dilantin), it may be necessary to utilize these designs since it is important to assess individual bioequivalence for these products.

They are several reasons that one may perform a logarithmic transformation of pharmacokinetic parameters. Westlake [58] and Rodda [11] discussed the

rationale for performing this transformation. However, the Biopharmaceutics Division of the FDA prefers to see both transformed and untransformed analyses while the Bioequivalence Division only examines the transformed analysis. If the analysis is performed based on the logarithmic transformation, an antilogarithmic transformation must be applied to transform the the confidence limits back to the original scale. In addition, the acceptable lower (upper) limit under an antilogarithmic transformation is 80% (125%). Liu and Weng [59] indicated that the exponentiation of the log-transformed confidence interval is shorter than the traditional confidence interval in general.

If the underlying distribution is not normal, other transformations, in addition to the logarithmic transformation, may also make the original distribution more symmetrical, such that one can still use the sample mean. However, an inappropriate transformation may often produce an incorrect estimate. Another approach is to use nonparametric methods to examine the robustness of the results. Several nonparametric methods have been used for crossover studies by Koch [60], Hauschke et al. [61], and Pabst and Jaeger [62]. Generally, these methods are based on the rank transformation and useful for hypothesis testing; however, they are not appropriate for estimation because, clinically, it is difficult to develop a decision rule based on ranks.

Since current bioequivalence criteria are derived from comparisons of average bioavailability (population bioequivalence), the majority of publications discussing designs and analysis of bioavailability trials focus on this topic. These include the work done by Westlake [56], Rodda [11], Chow and Liu [63], and Weiner and Yuh [3]. To ensure that patients can switch from one formulation to another (e.g., a generic brand), population equivalence is not sufficient to provide a good estimate for individual equivalence. Thus, one should use the rules that can assess individual bioequivalence. However, these methods still await a clinical decision criterion that will be generally accepted by regulatory agencies and the scientific community. There is also a need to develop a procedure to handle experimental designs with more than two treatment groups. As expected, these methods require a large sample size in order to make a meaningful assessment. Also note that one may have to use extended period designs in order to use these methods. However, it is suggested that one should try at least one simple alternative approach (e.g., calculate the concordance correlation). If the result is different from the traditional population equivalence method, an extended period crossover design should be used to examine the new formulation more carefully.

If a drug product has active metabolites and/or there are more than two formulations in a bioavailability trial, an adjustment for multiple comparisons has to be made for the confidence interval of each pharmacokinetic parameter. However, most classic

adjustments are constructed to adjust the false-positive rate (type I error) that will always widen the confidence interval. Procedures

such as the one suggested by Giani and Strassburger [64] could be modified and applied to bioavailability trials.

It appears that there are still many outstanding statistical issues. An optimal procedure has to be statistically sound and clinically relevant. Thus, based on both theoretical and practical considerations, clinicians, statisticians, and pharmacokineticists from industry, academia, and government must work together to develop a strategy for the design, analysis, and acceptance criteria for bioavailability studies.

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References

1. Food and Drug Administration. Bioavailability and bioequivalence requirements. Fed. Reg. 42:16381643, 1977.

2. Food and Drug Administration. Fed. Reg. 42:16241653, 1977.

3. D. L. Weiner and L. Yuh. Bioavailability studies. In Statistics in the Pharmaceutical Industry. Edited by C. R. Buncher and J. Tsay. Marcel Dekker, New York, 1994, pp. 215245.

4. M. N. Martinez and A. J. Jackson. Suitability of various noninfinity area under the plasma concentration-time curve (AUC) estimates for use in bioequivalence determinations: relationship to AUC from zero to time infinity (AUC 0-inf). Pharm. Res. 4:512517, 1991.

5. A. J. Jackson and M. Chen. Application of moment analysis in assessing rates of absorption for bioequivalency studies. J. Pharm. Sci. 76:69, 1987.

6. M. Chen and J. Jackson. The role of metabolites in bioequivalency assessment. I. Linear pharmacokinetics without first-pass effect. Pharm. Res., 8:2532, 1991.

7. W. J. Westlake. The use of balanced incomplete block designs in comparative bioavailability trials. Biometrics 30:319329, 1974.

8. L. Yuh and S. J. Ruberg. Latin square designs in a comparative bioavailability study. Drug Inf. J. 24:289297, 1990.

9. G. Ekbohm and H. Melander. The subject-by-formulation interaction as a criterion of

interchangeability of drugs. Biometrics 45:12491254, 1989.

10. S. Hwang, P. B. Huber, M. Hesney and K. C. Kwan. Bioequivalence and interchangeability. J. Pharm. Sci. 67:4, 1978.

11. B. E. Rodda. In Bioavailability: Design and Analysis in Statistical Methodology in the Pharmaceutical Sciences. Edited by D. A. Berry. Marcel Dekker, New York, 1989, pp. 5781.

12. K. F. Phillips. Power of the two one-sided test procedure in bioequivalence. J. Pharmacokinet. Biopharm. 18:137144, 1990.

13. D. Z. D'Argenio. Optimal sampling times for pharmacokinetic experiments. J. Pharmacokinet. Biopharm. 9:739755, 1981.

14. J. J. DiStefano, III. Optimized blood sampling protocols and sequential design of kinetic experiments. Am. J. Physiol. 240:259265, 1981.

15. J. J. DiStefano, III. Algorithms, software and sequential optimal sampling schedule designs for pharmacokinetics and physiologic experiments. Math. Comput. Simul. 24:531534, 1982.

16. D. Katz and D. D'Argenio. Experimental design for estimating integrals by numeric quadrature, with applications to pharmacokinetic studies. Biometrics 39:621628, 1983.

17. G. L. Drusano, A. Forrest, M. J. Snyder, M. D. Reed, and J. L. Blumer. An evaluation of optimal sampling strategy and adaptive study design. Clin. Pharmacol. Ther., 44:232238, 1988.

18. E. M. Landaw. Optimal multicompartmental sampling designs for parameter estimation: practical aspects of the identification problem. Math. Comput. Simul., 24:525530, 1982.

19. T. A. Bancroft. Analysis and inference for incompletely specified models involving the use of preliminary tests of significance. Biometrics 20:427442, 1964.

20. W. J. Westlake. Use of confidence intervals in analysis of comparative bioavailability trials. J. Pharm. Sci. 61:13401341, 1972.

21. C. M. Metzler. Bioavailability: a problem in equivalence. Biometrics 30:309317, 1974.

22. E. Shirley. The use of confidence intervals in biopharmaceutics. J. Pharm. Pharmacol. 28:312313, 1976.

23. C. S. Locke. An exact confidence interval from untransformed data for the ratio of two formulation means. J. Pharmacokinet. Biopharm. 12:649655, 1984.

24. D. J. Schuirmann. Confidence intervals for the ratio of two means from a crossover study. Proc. 1989 Biopharmaceutical Sect. Am. Stat. Assoc. 121126, 1990.

25. W. J. Westlake. Symmetric confidence intervals for bioequivalence trials. Biometrics 32:741744, 1976.

26. N. Mantel. Do we want confidence intervals symmetrical abut the null value? Biometrics 33:759760, 1977.

27. D. Mandallaz and J. Mau. Comparison of different methods for decision making in

bioequivalence assessment. Biometrics 37:213222, 1981.

28. V. W. Steinijans and E. Dilleti. Statistical analysis of bioavailability studies: parametric and nonparametric confidence Intervals. Eur. J. Clin. Pharmacol. 24:127136, 1983.

29. T. B. L. Kirkwood. Bioequivalence testinga need to rethink. Biometrics 37:589, 1981.

30. C. M. Metzler. Statistical methods for deciding bioequivalence of formulations. In Oral Sustained Release Formulation: Design and Evaluation. Edited by A. Yacobi, and E. Halphin-Walega. Pergamon Press, New York, 1988, pp. 217238.

31. Food and Drug Administration. Report by the Bioequivalence Task Force on recommendations from the bioequivalence hearing conducted by the FDA. FDA, Washington, D.C., 1988.

32. E. L. Lehmann. Testing Statistical Hypotheses. John Wiley & Sons, New York, 1959.

33. W. H. Bondy. A test of an experimental hypothesis of negligible difference between means. Am. Stat. 23:38-30, 1969.

34. S. Anderson and W. W. Hauck. A new procedure for testing equivalence in comparative bioavailability and other clinical trials. Commun. Stat. A12:26632692, 1983.

35. D. M. Rocke. On testing for bioequivalence. Biometrics 40:225230, 1984.

36. B. E. Rodda and R. L. Davis. Determine the probability of an important difference in bioavailability. Clin. Pharmacol. Ther. 28:247252, 1980.

37. M. R. Selwyn, A. P. Dempster, and N. R. Hall. A Bayesian approach to bioequivalence for the 2×2 changeover design. Biometrics 37:1121, 1981.

38. M. R. Selwyn and N. R. Hall. On Bayesian methods for bioequivalence. Biometrics 40:11031108, 1984.

39. H. P. Wijnand, H. P. and C. J. Timmer. Mini-computer programs for bioequivalence testing of pharmaceutical drug formulations in two-way crossover studies. Comput. Prog. Biomet. 17:7378, 1983.

40. A. E. Gelfand, S. E. Hills, A. Racine-Poon, and A. F. M. Smith. Illustration of Bayesian inference in normal data models using Gibbs sampling. J. Am. Stat. Assoc. 85:972985, 1990.

41. D. J. Schuirmann. A comparison of two one-sided tests procedure and the power approach for assessing the equivalence of average bioavailability. J. Pharmacokinet. Biopharm. 15:657680, 1987.

42. C. M. Metzler. Bioavailability/bioequivalence: study design and statistical issues. J. Clin. Pharmacol. 29:289292, 1989.

43. L. Yuh. On robust estimation in bioavailability/bioequivalence studies. Proceedings, Bio-International '89, Toronto, Canada, 1990, pp. 162165.

44. L. Yuh and S. J. Ruberg. On robust estimation using individual ratios in bioavailability trials. Commun. Stat. A23:545554, 1994.

45. S. Anderson and H. H. Hauck. Consideration of individual bioequivalence. J. Pharmacokinet. Biopharm. 18:259274, 1990.

46. Food and Drug Administration. Fed. Reg. 43:6968, 1978.

47. J. D. Haynes. Statistical stimulation study of new proposed uniformity requirement

for bioavailability studies. J. Pharm. Sci. 70:673675, 1981.

48. C. M. Metzler and D. C. Huang. Statistical methods for bioavailability. Clin. Res. Pract. Drug. Reg. Affairs. 1:109132, 1983.

49. T. W. Dobbins and B. Thiyagrajan. A retrospective assessment of the 75/75 rule in bioequivalence. 11:13331342, 1992.

50. E. J. G. Pitman. A note on normal correlation. Biometrika 31:912, 1939.

51. W. A. Mogan. A test for the significance of the difference between the two variables in a sample from a normal bivariate population. Biometrika 31:1319, 1939.

52. L. Yuh. On assessing bioequivalence using the concordance correlation coefficient. Proceedings, Bio-International '92, Bad Homburg, Germany. Edited by K. K. Midha and H. H. Blume. Medpharm GmbH Scientific Publishers, Stuttgart, Germany, 1993, pp. 283288.

53. L. B. Sheiner. Bioequivalence revisited, Stat. in Med. 11:17771788, 1992.

54. R. Schall and H. Luus. On population and individual bioequivalence. Stat. Med. 12:11091124, 1993.

55. M. Chen. An alternative approach for assessment of rate of absorption in bioequivalence studies. Pharm. Res. 9:13801385, 1992.

56. T. N. Tozer. Metrics of absorption: simulation approach. Presented at Bio-International '94, Munich, Germany, 1994.

57. L. F. Lacey, O. N. Keene, C. Duquesnoy, and A. Bye. Evaluation of different indirect measures of rate of drug absorption in comparative pharmacokinetic studies. J. Pharm. Sci. 83:212215, 1994.

58. W. J. Westlake. Bioavailability and bioequivalence of pharmaceutical formulations. In Biopharmaceutical Statistics for Drug Development. Edited by K. E. Peace. Marcel Dekker, New York, 1988, pp. 329352.

59. J.-P. Liu and C.-S. Weng. Evaluation of log-transformation in assessing bioequivalence. Commun. Stat. A23:421434, 1994.

60. G. G. Koch. The use of nonparametric methods in the statistical analysis of two-period changeover design. Biometrics 28:577584, 1972.

61. D. Hauschke, V. W. Steinjans, and E. Diletti. A distribution-free procedure for the statistical analysis of bioequivalence studies. Int. J. Clin. Pharmacol. Ther. Toxicol. 28:7278, 1990.

62. G. Pabst and H. Jaeger. Review of methods and criteria for the evaluation of bioequivalence studies. Eur. J. Clin. Pharmacol. 38:510, 1990.

63. S. C. Chow and J.-P. Liu. Design and Analysis of Bioavailability and Bioequivalence Studies. Marcel Dekker, New York, 1992.

64. G. Giani and K. Strassburger. Testing and selecting for equivalence with respect to a control. J. Am. Stat. Assoc. 89:320329, 1994.

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